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VOLUME 90

FEBRUARY TO JUNE, 1946

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $1.75. Subscription per volume (three issues), $4.50.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between July 1 and September 1, and to the Department of Zoology, Washington University, St. Louis, Missouri, during the remainder of the year.

Entered as second-class matter May 17, 1930, at the post office at Lancaster, Pa., under the Act of August 24, 1912.

LANCASTER PRESS, INC., LANCASTER, PA.

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Biological Bulletin

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Vol. 90, No. 1 February, 1946

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

# STUDIES ON CILIATES OF THE FAMILY ANCISTROCOMIDAE CHATTON AND LWOFF (ORDER HOLOTRICHA, SUBORDER THIGMOTRICHA)

## I. HYPOCOMINA TEGULARUM SP. NOV. AND CREBRICOMA GEN. NOV.

EUGENE N. KOZLOFF

*Lewis and Clark College, Portland, Oregon*

### Introduction

The genus *Hypocomina* was proposed by Chatton and Lwoff (1924) to include a single species, *Hypocoma patellarum* Lichtenstein (1921), parasitic on the pallial branchiae of *Patella caerulea* L. Subsequently, Raabe (1934) described as *Hypocomina carinata* a ciliate from *Mytilus edulis* L. which Jarocki (1935) pointed out obviously does not belong to the genus *Hypocomina*. Raabe used the name *Hypocomina carinata* again in 1938, however, and no literature has come to my attention in which the form in question is assigned to another genus. In the present paper I will describe as *Hypocomina tegularum* sp. nov. an ancistrocomid ciliate from the ctenidium of *Tegula brunnea* (Philippi); and on the basis of my studies on the morphology of a ciliate which I presume to be identical with *"Hypocomina" carinata*, I will establish the position of Raabe's species in a new genus, *Crebricoma*.

### Hypocomina tegularum sp. nov.

(Plate I, Figs. 4, 5)

The body is elongated and compressed dorso-ventrally. Fifteen living individuals taken at random ranged in length from 26 $\mu$ to 36 $\mu$, in width from 12 $\mu$ to 17 $\mu$, and in thickness from 9 $\mu$ to 12 $\mu$, averaging about 31 $\mu$ by 15 $\mu$ by 11 $\mu$. As seen in dorsal view the curvature of the left margin of the ciliate is a little more pronounced than that of the right margin, although this asymmetry is not conspicuous and in most fixed specimens is barely apparent. The body is widest near the middle and is rounded posteriorly. The anterior end is attenuated, bent ventrally, and truncate at the tip. The reduced ciliary system, to be described presently, is disposed in a shallow, relatively flat depression occupying the anterior half of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the thigmotactic field are convex.

A contractile suctorial tentacle enables the ciliate to attach itself to the epithelial cells of the ctenidium of the host and to suck out their contents. I have not yet succeeded in determining the exact nature of the tentacle in the ancistrocomid ciliates which I have examined and the accounts of other authors regarding its structure are likewise unsatisfactory. In *Hypocomina tegularum*, it appears to be a membranous tube-like extension of the attenuated anterior end which is protracted and contracted at will. When fully extended the tentacle is about 3 $\mu$ in length and 1–1.5 $\mu$ in diameter. It is hoped that further studies on ancistrocomid ciliates will shed some light on the problem of the structure of this interesting organelle.

The internal tubular canal continuous with the suctorial tentacle is difficult to study in the living ciliates, but in material stained with iron hematoxylin or alum hematoxylin it can usually be traced posteriorly for a distance equal to about one-third the length of the body (Plate I, fig. 4, c). In most individuals it is directed obliquely to the right. In fixed specimens the extreme anterior portion of the canal is approximately 1–1.5 $\mu$ in diameter, while the remainder of it appears to be swollen and to have the form of a poorly defined clear area. Lichtenstein (1921) interpreted the comparable swelling of the canal in *Hypocomina patellarum* to be a "balle alimentaire." Jarocki (1935) attributed the clear area described by Raabe (1934) behind the tentacle of *Hypocomella macomae* and *"Hypocomina" carinata* to hyperdistension of the canal due to unequal infiltration by the fixing reagent or as a result of its action upon moribund, partly plasmolyzed individuals. It is interesting to note in this connection, however, that some ancistrocomid ciliates rarely show this phenomenon upon fixation and the canal can be traced to within a short distance of the posterior end of the body.

The delicate thigmotactic cilia of *Hypocomina tegularum* are about 6–7 $\mu$ in length and are disposed in nine longitudinal rows. All the rows originate about 3 $\mu$ or 4 $\mu$ posterior to the base of the suctorial tentacle and are about one-half the length of the body (Plate I, fig. 4). The first five rows from the right side of the ciliate are appreciably longer and sometimes appear to lie closer together than the other four rows. Chatton and Lwoff (1924) reported that in *Hypocomina patellarum* a ridge-like eminence (carène) divides the ciliary area into two unequal fields. These authors did not, however, state how many of the ten ciliary rows described by Lichtenstein (1921) for *H. patellarum* are in each complex and did not give any facts concerning the relative lengths of the rows comprising the two fields. In *Hypocomina tegularum* I have not discerned an unmistakable eminence separating the five rows on the right from the four rows on the left, although in some living and fixed individuals the distance between the distal portions of the fifth and sixth rows is somewhat greater than the distance between the other rows.

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EXPLANATION OF PLATE I

FIGURE 1. *Crebricoma carinata* (Raabe) comb. nov. Ventral aspect. Schaudinn's fixative-iron hematoxylin. Drawn with aid of camera lucida. × 1130.

FIGURE 2. *Crebricoma carinata* (Raabe) comb. nov. Dorsal aspect, from life.

FIGURE 3. *Crebricoma carinata* (Raabe) comb. nov. Lateral aspect from right side, from life.

FIGURE 4. *Hypocomina tegularum* sp. nov. Ventral aspect. Schaudinn's fixative-iron hematoxylin. Drawn with aid of camera lucida. × 1870.

FIGURE 5. *Hypocomina tegularum* sp. nov. Lateral aspect from left side, from life.

c = internal tubular canal, cv = contractile vacuole, fv = food vacuole, ma = macronucleus, mi = micronucleus.

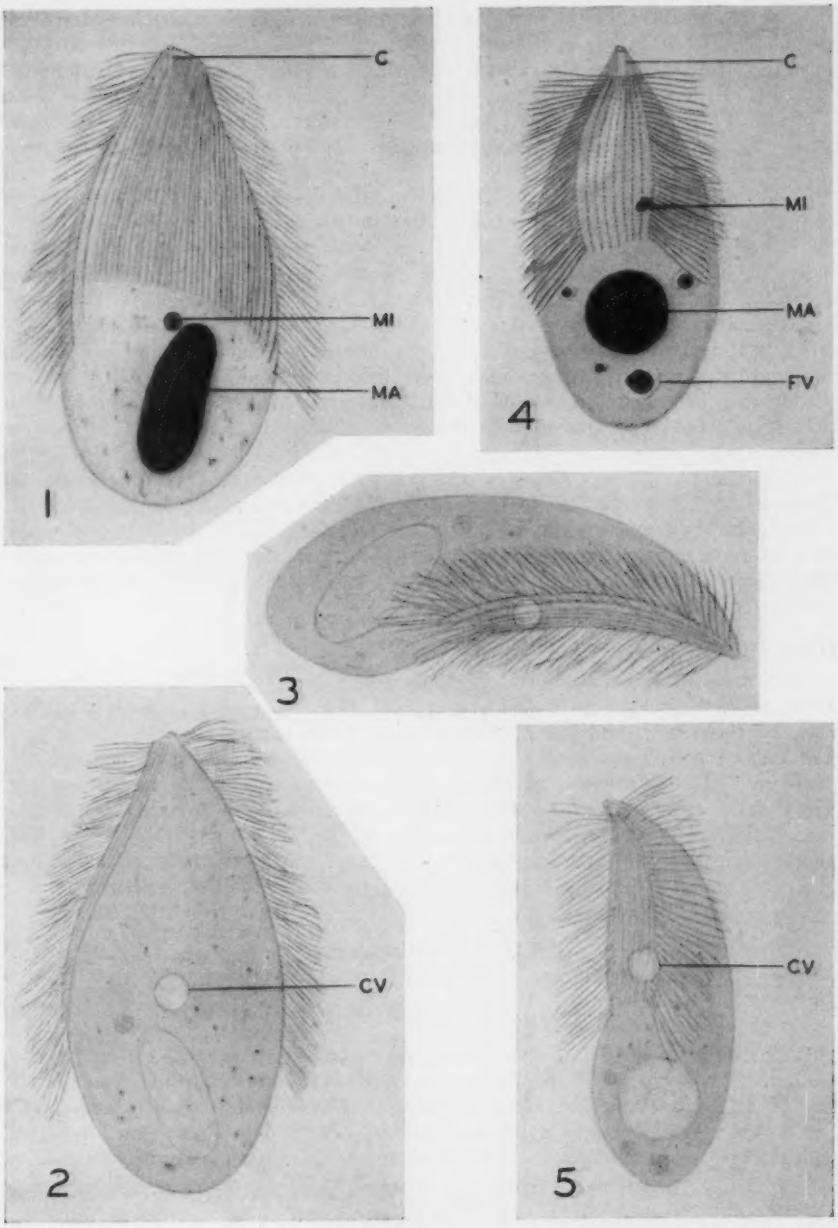


PLATE I

The cytoplasm is colorless and contains numerous food inclusions and refractile granules. The refractile granules are apparently lipoid droplets, as they are dissolved out by toluol used for clearing following staining. One or more large food vacuoles (Plate I, fig. 4, fv) are usually present in the posterior part of the body. The contractile vacuole (Plate I, fig. 5, cv) lies near the middle of the body and opens to the exterior on the ciliated ventral surface. I have not detected a permanent opening in the pellicle.

The spherical macronucleus (Plate I, fig. 4, ma) is situated in the posterior half of the body. In preparations stained with iron hematoxylin or alum hematoxylin the chromatin appears to be aggregated into many large, round granules. In ten individuals fixed in Schaudinn's fluid and stained with iron hematoxylin the diameter of the macronucleus ranged from 5.5 $\mu$ to 7 $\mu$.

The spherical or ovoid micronucleus (Plate I, fig. 4, mi) is usually situated anterior to the middle of the body. It is very difficult to detect in living individuals. In stained preparations the chromatin is dispersed into granules of varying size and shape lying for the most part near the periphery. In ten individuals fixed in Schaudinn's fluid and stained with iron hematoxylin the diameter of the micronucleus ranged from 1.6 $\mu$ to 2.2 $\mu$.

I found *Hypocomina tegularum* to be present in small numbers on the ctenidium of two of three specimens of *Tegula brunnea* (Philippi) which I collected at Carmel Bay, California, in September, 1944.[1] Since that time I have had the opportunity to examine a large number of individuals of *Tegula brunnea* from localities near Moss Beach and Dillon Beach, California, but found none to be infested by *Hypocomina tegularum*.

*Hypocomina tegularum sp. nov.*

Diagnosis: Length 26 $\mu$–36 $\mu$, average about 31 $\mu$; width 12 $\mu$–17 $\mu$, average about 15 $\mu$; thickness 9 $\mu$–12 $\mu$, average about 11 $\mu$. The anterior end is attenuated, bent ventrally, and provided with a contractile suctorial tentacle continuous with an internal tubular canal. The ciliary rows are nine in number and are disposed in a shallow depression occupying the anterior half of the ventral surface. The first five rows from the right are slightly longer than the other four rows. The macronucleus is spherical and is situated in the posterior half of the body. The micronucleus lies anterior to the middle of the body. Ectoparasitic on the ctenidium of *Tegula brunnea* (Philippi) (Carmel Bay, California). Syntypes are in the collection of the author.

## CREBRICOMA GEN. NOV.
## CREBRICOMA CARINATA (RAABE) COMB. NOV.

(Figure 1. Plate 1, Figs. 1–3)

I have distinguished two species of ancistrocomid ciliates parasitic on the epithelium of the palps and ctenidial filaments of *Mytilus edulis* L. from various localities in San Francisco Bay. One of these is *Hypocomides mytili* Chatton and Lwoff; the other I presume to be identical with *"Hypocomina" carinata* Raabe, for which I propose a new genus, *Crebricoma*. My observations on the morphology

[1] I am indebted to Dr. D. T. MacDougal for his kindness in arranging my trip to Carmel which made possible the collection of the original material of the ciliate described herein.

of this species permit me to augment the original description given by Raabe. It seems advisable, therefore, to present herein a revised description of *Crebricoma carinata*.

The body is elongated and somewhat compressed dorso-ventrally. Fifteen living individuals taken at random ranged in length from 58 $\mu$ to 71 $\mu$, in width from 27 $\mu$ to 39 $\mu$, and in thickness from 22 $\mu$ to 31 $\mu$, averaging about 64 $\mu$ by 31 $\mu$ by 25 $\mu$. The anterior end is narrowed, bent ventrally, and in dorsal view is seen to end in an oblique truncation, the right side of which projects a little more than the left side (Plate I, figs. 1, 2). The body is widest near the middle and is rounded posteriorly. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior two-thirds of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the thigmotactic area are convex.

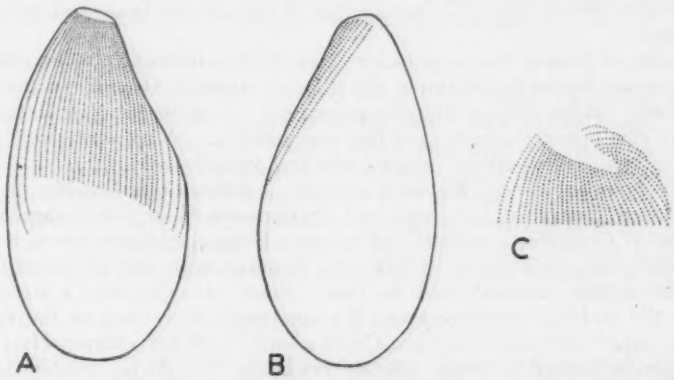


FIGURE 1. *Crebricoma carinata* (Raabe) comb. nov. Distribution of ciliary rows. Semi-diagrammatic representations based on camera lucida drawings of specimens fixed in Schaudinn's fluid and impregnated with activated silver albumose (protargol).
A. Ventral aspect, B. Dorsal aspect, C. View of anterior end.

The suctorial tentacle is situated on the right side of the anterior truncation. I am at present unable to make any conclusive statement with regard to the structure and contractile properties of the tentacle in this species. The internal tubular canal continuous with the tentacle cannot be satisfactorily distinguished in living material, but can be demonstrated in some fixed specimens which have been stained with iron hematoxylin. It appears to pass at first dorsally and then ventrally and obliquely to the right. I have not succeeded in tracing it posteriorly for more than a short distance. A broad lighter area behind the anterior part of the canal is frequently observed in fixed individuals.

As described by Raabe, the ciliary system of *Crebricoma carinata* consists of about twenty closely-set rows bordered on the right by two longer and more widely-spaced rows and on the left by three such rows. According to my observations, however, the more widely-spaced rows on the left side do not represent a complex separate from the closely-set rows. The number of rows in the thigmotactic field

of *C. carinata* is very difficult to determine, since some of the rows on the left are lateral in position and several of them originate on the dorsal surface. In one individual impregnated with activated silver albumose I counted thirty-four rows and in another individual, thirty-six rows. In all specimens which I have examined carefully the number of ciliary rows exceeded thirty-two.

The two widely-spaced rows on the right side of the thigmotactic field originate on the ventral surface near the base of the suctorial tentacle. The outer row is the longer and is about two-thirds the length of the body (Fig. 1A). All the rows of the second complex, with the exception of three or four rows on the extreme left which are about as widely-spaced as the two long rows on the right side, are very closely-set and are one-half to two-thirds the length of the body, becoming progressively longer toward the left. Several of the rows on the left side of the thigmotactic field originate on the left lateral margin or on the dorsal surface (Fig. 1B; Plate I, fig. 2). The arrangement of the ciliary rows at the anterior end thus forms an incomplete suture (Fig. 1C) reminiscent of the anterior field of other holotrichous ciliates.

The cilia of *Crebricoma carinata* are about 10–11 $\mu$ in length. When the organism is attached to the epithelium of the palps or ctenidial filaments of the host the cilia usually exhibit only a sluggish movement. Occasionally one or two small groups of cilia near the anterior end beat energetically. When dissociated from the host *C. carinata* swims actively and its cilia beat metachronously.

Raabe proposed the specific name *carinata* in allusion to a keel-like prominence on the dorsal surface. I have seen such prominences on seriously shrunken fixed specimens of *Crebricoma carinata* and on some living individuals which were obviously undergoing plasmolysis. I have not, however, been able to distinguish them on ciliates freshly removed from the host. Raabe also described a sunken space between the keel-like prominence and the outermost ciliary row on the right side, which he said "vermutlich dem von Chatton und Lwoff bei anderen Hypocomiden beschriebenen 'vestige de frange adorale' entspricht." I do not believe such a depression exists in normal individuals, although I have observed groove-like and slit-like depressions form on the lateral and dorsal surfaces of moribund specimens of *Crebricoma carinata,* as well as *Hypocomides mytili.*

The cytoplasm is colorless and contains a few small refractile granules, which presumably are lipoid droplets, and food inclusions. I have not discerned any large food vacuoles in this species. The contractile vacuole (Plate I, fig. 2, cv) opens to the exterior on the ventral surface near the middle of the body. There appears to be no permanent opening in the pellicle.

The sausage-shaped or ovoid macronucleus (Plate I, fig. 1, ma) is situated in the posterior half of the body. In preparations stained with iron hematoxylin or the Feulgen nuclear reaction the chromatin is aggregated into a dense reticulum enclosing vacuole-like clear spaces of varying size. Viewed dorsally, the longitudinal axis of the macronucleus is ordinarily placed obliquely to the longitudinal axis of the body. In most specimens the anterior end of the macronucleus is directed dorsally, while the posterior end is directed ventrally. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen nuclear reaction the macronucleus ranged in length from 13 $\mu$ to 24.3 $\mu$ and in width from 5.6 $\mu$ to 11.7 $\mu$.

The spherical micronucleus (Plate I, fig. 1, mi) is readily detected in the living ciliate. It is commonly situated near the dorsal surface close to the anterior end of

the macronucleus. Following fixation the micronucleus stains lightly with iron hematoxylin and the Feulgen reaction. The chromatin appears to be homogeneous. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from 2.7 $\mu$ to 3.6 $\mu$.

*Crebricoma gen. nov.*

Diagnosis: The body is elongated, somewhat flattened dorso-ventrally, and narrowed anteriorly. The anterior end is provided with a contractile suctorial tentacle continuous with an internal tubular canal. The ciliary rows are numerous and except for some which originate on the dorsal surface or left lateral margin are disposed on the ventral surface. Two long, widely-spaced rows on the right side of the ciliary system appear to form a complex separate from the remaining rows, which are for the most part closely-set. The arrangement of the ciliary rows at the anterior end of the body forms an incomplete suture. Genotype: *Crebricoma carinata* (Raabe) comb. nov. (= *Hypocomina carinata* Raabe).

*Crebricoma carinata (Raabe) comb. nov.*

Diagnosis: Length 58 $\mu$–71 $\mu$, average about 64 $\mu$; width 27 $\mu$–39 $\mu$, average about 31 $\mu$; thickness 22 $\mu$–31 $\mu$, average about 25 $\mu$. Two widely-spaced rows of cilia on the right side, the outer of which is about two-thirds the length of the body, and a series of more than thirty rows, which with the exception of three or four on the left are closely-set, comprise the ciliary system. The closely-set rows are one-half to two-thirds the length of the body, becoming progressively longer toward the left. The macronucleus is sausage-shaped or ovoid. The micronucleus is spherical. Ectoparasitic on the palps and branchial filaments of *Mytilus edulis* L.

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# OBSERVATIONS ON THE GIANT AMOEBA, AMOEBA CAROLINENSIS (WILSON, 1900) [1]

ROBERT B. SHORT

*Miller School of Biology, University of Virginia*

## Introduction

During the past few years there has been considerable controversy over the name of the giant fresh-water amoeba which Wilson (1900) described and named *Pelomyxa carolinensis*. He placed this amoeba in the genus *Pelomyxa* evidently because it has many nuclei.

Mast (1938) and Rice (1945) agree with Wilson in regard to the name of this form. The latter author says that the principal characteristic of the genus *Pelomyxa* is its many nuclei. Greeff (1874), who established the genus, states that besides vacuoles there are in *Pelomyxa palustris* three kinds of characteristic structures: (1) Nuclei, (2) Hyaline and homogeneous bodies of spherical, ellipsoidal, or irregular shape, and (3) *Stäbchen*, little rods (later considered to be symbiotic bacteria). He does say that the large number of nuclei forms *a* principal characteristic of the genus *Pelomyxa*. However, since Greeff's description of *P. palustris*, pelomyxae have been described with one or few nuclei (*P. Belevskii* Penard, 1888; *P. binucleata* (Gruber, 1885) Penard, 1902; *P. paradoxa* Penard, 1902; *P. lentissima* Schaeffer, 1918; *P. schiedti* Schaeffer, 1918), and amoeboid forms other than pelomyxae have been described with more than one nucleus. *Amoeba proteus* has been observed in this laboratory with as many as eight nuclei.[2] Also the hyaline spherical bodies are not found exclusively in *Pelomyxa*. In view of these facts, Penard (1902) states that the chief criterion for the genus *Pelomyxa* is the presence of symbiotic bacteria in the cytoplasm. He says, "S'il me fallait donc caractériser le genre Pelomyxa, je le ferais à peu près en ces terms: 'Amibes à mouvements lents, toujours pourvues de bactéries symbiotiques.'" He then points out that Wilson's rhizopod has in common with the *Pelomyxa* only the *Glanzkörper* and that this form has nothing to distinguish it from the genus *Amoeba*.

Schaeffer (1918) in describing *Pelomyxa lentissima* states, "Other inclusions in the ectoplasm are the bacterial rods, distinctive of the genus." Later in his account of *P. schiedti* he says, "The bacterial rods, the presence of which characterizes the genus *Pelomyxa*, are found in considerable numbers in *schiedti*." Bourne (1891) in speaking of *P. viridis* describes the organism as, "densely packed with bacteria."

Thus it is seen that the chief characteristic of the genus *Pelomyxa* is the presence of symbiotic bacteria in the cytoplasm. Since the giant amoeba does not possess these bacteria, it is not a *Pelomyxa*.

[1] The author wishes to express his grateful appreciation to Dr. B. D. Reynolds for his suggestions and helpful advice in regard to this work and to thank Dr. C. M. Gilbert for help in taking the photomicrographs.

[2] Personal communication from Dr. B. D. Reynolds.

Schaeffer (1926, 1938a) maintains that Wilson's amoeba represents Roesel's (1755) "der kleine Proteus," and should therefore be called *Chaos chaos* Linnaeus. But Roesel's description is entirely inadequate to establish a species or even a genus. He undoubtedly had an amoeboid form, but it is impossible to find out the exact structure of his "Proteus." He mentions granules (*Körnern*) but says nothing of vacuoles, which in the giant amoeba are much larger than the crystals (granules). Schaeffer seems to base his conclusion mainly on the size and shape of the "Proteus." He contends that Roesel was a reliable investigator and that his figures may therefore be credited with general accuracy. As Schaeffer (1926) points out, "Roesel states the natural size of his amoeba in the rounded (spherical?) form to have been the same as figure A, which measures about 1660 $\mu$ in diameter." Yet Schaeffer (1938a) says, "*Chaos chaos* has a distinctive size range which fluctuates around 500 $\mu$ diameter." Thus, Schaeffer himself gives the diameter of the giant amoeba as about $\frac{1}{3}$ that of Roesel's "Proteus." There are also striking differences between Roesel's figures and description of a binary fission in his "Proteus" and Schaeffer's (1938b) photographs and account of the "3-daughter division of the giant amoeba."

Mast and Johnson (1931) review the data presented by Roesel and express the opinion that the latter possibly was dealing with a myxomycete. Rice (1945) states, "It is impossible to ascertain the exact structure of Roesel's 'der kleine Proteus.'" Thus since both the generic and specific names, *Chaos chaos*, are based on Roesel's inadequate description, they are not valid.

Hegner and Taliaferro (1924) in referring to Wilson's large amoeba use the name *Amoeba carolinensis* for it, though they give no reason for doing so. The present investigation adds evidence to support the use of this name. Therefore, *Amoeba carolinensis* (Wilson, 1900) Hegner and Taliaferro, 1924 is considered to be the correct name of the organism used in these experiments.

## CYTOLOGY AND PHYSIOLOGY

### Materials and Methods

The original stock culture of *Amoeba carolinensis* was procured from the General Biological Supply House, Chicago, Illinois, in November 1944. About every two weeks subcultures were made in the following manner: Spring water containing five wheat grains per 100 cc. of water was boiled for a few minutes and left uncovered for several days. One or two of the grains of wheat were put into a butter dish and about 90 cc. of the boiled water was poured in, giving a depth of $\frac{3}{4}$ inches. The amoebae along with food organisms, which consisted chiefly of rotifers, *Chilomonas paramecium*, *Colpidium colpoda*, and *Paramecium caudatum*, were then added. In the best cultures a water mold grew on the wheat grains and many amoebae often were found among the hyphae of the mold. These stock cultures had been maintained for a little more than four months when the experiments began.

In addition to the wheat culture medium (W. C. M.) a hay medium (H. C. M.) was made as follows: 10 grams of chopped timothy hay were added to 1000 cc. of spring water and boiled for about 15 minutes. The solution was filtered to remove the solid particles and then put into test tubes with cotton stoppers and autoclaved on two successive days at 10 pounds pressure. From these test tubes the medium

was taken to start new cultures during the experiments. After trying various concentrations of this hay medium it was found that the amoebae and food organisms remained healthier and increased more rapidly if the stock solution was diluted with four parts of boiled and aerated spring water.

Cultures of *Chilomonas paramecium* and *Paramecium caudatum* were established in both the wheat and the hay culture media to serve as food organisms for the amoebae during the experiments. Subcultures were made from these about every two weeks.

Forty specimens of *Amoeba carolinensis* were washed six times in boiled spring water. Ten were transferred to two stender dishes (five to each dish) containing paramecia in W. C. M. In a similar manner ten were transferred to two stender dishes containing chilomonads in W. C. M.; ten, to two stender dishes containing paramecia in H. C. M.; and ten, to two stender dishes containing chilomonads in H. C. M. Each day the cultures were inspected and the supply of food organisms was replenished if it was low. The amoebae were counted daily for seven-day periods and then on the seventh day (in some cases a few days later) new cultures were made from the ones of the preceding week, so that by the end of the experiment most of the amoebae had been growing in one kind of medium and feeding on one kind of food organism for at least 46 days. With the exception of two cultures, the pH was taken on each culture on or after the seventh day. During the experiment, observations as to the relative size and form of the amoebae were made; some of the amoebae in each culture were examined in hanging drops under the compound microscope; and at the end of the experiment photomicrographs were taken of a few amoebae in the wheat-culture medium.

## Results

After the first two weeks there were marked differences in the rates of reproduction, especially between those amoebae grown in W. C. M. and fed on paramecia and the other cultures, as can be seen in Table I. During the first two weeks there seems to be a period of adjustment when the reproduction rates of the different cultures do not vary significantly. However, during the third to sixth weeks the amoebae grown in W. C. M. and fed on paramecia increased rapidly—from 10 to 45 during the sixth week. The other cultures showed little or no growth.

The hydrogen-ion concentrations remained rather constant. The pH's varied from 7.0 to 8.1, with that of the H. C. M. containing paramecia usually a little higher than the others. The room temperature varied from about 20° to 26° C.

There was no significant difference in size of the amoebae in the various cultures, but there was a difference in form. Those growing in W. C. M. and fed on paramecia were often somewhat disc-shaped with short, blunt pseudopods radiating from all sides as shown in Figure 1a. Others were usually monopodal or bipodal with blunt pseudopods as shown in Figure 1b. Clumping of the disc-shaped amoeba was observed five or six times.

The amoebae in W. C. M. fed on chilomonads were more flattened, with thinner pseudopods which were often at right angles to the main part of the body and frequently branched (Fig. 1c). The pseudopods often had little knob-like swellings at their distal ends (Fig. 1d). The amoebae in these cultures occasionally were monopodal in form.

TABLE I

Effect of culture media and food organisms on the reproduction rates of *Amoeba carolinensis*. At the beginning of each week amoebae were put into new cultures corresponding to the conditions under which they previously had been growing, so that the end results represent cumulative effects. Since there always were two cultures of each type, the pH's of both cultures are given.

| | Wheat medium with paramecia | | | | Wheat medium with chilomonads | | | | Hay medium with paramecia | | | | Hay medium with chilomonads | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Number of amoebae | | % increase | pH | Number of amoebae | | % increase | pH | Number of amoebae | | % increase | pH | Number of amoebae | | % increase | pH |
| | First day | Seventh day | | | First day | Seventh day | | | First day | Sixth day | | | First day | Seventh day | | |
| First week | 10 | 17 | 70% | 7.5<br>7.6 | 10 | 22 | 120% | 7.5<br>7.5 | 10 | 18 | 80% | 7.7<br>7.8 | 10 | 11 | 10% | 7.5<br>7.5 |
| | | | | | | | | | | Seventh day | | | | | | |
| Second week | 10 | 15 | 50% | 7.6<br>7.6 | 9 | 14 | 55½% | 7.5<br>7.6 | 10 | 11 | 10% | 8.0<br>7.9 | 10 | 10 | 0% | 7.8<br>7.7 |
| Third week | 10 | 30 | 200% | | 10 | 10 | 0% | 7.8<br>7.9 | 10 | 16 | 60% | 8.0<br>8.1 | 10 | 10 | 0% | 8.0<br>8.1 |
| Fourth week | 10 | 48 | 380% | 7.0<br>7.1 | 10 | 10 | 0% | 7.6<br>7.6 | 10 | 10 | 0% | 7.6<br>7.4 | 10 | 11 | 10% | 7.9<br>8.0 |
| Fifth week | 10 | 57 | 470% | 7.5<br>7.4 | 10 | 13 | 30% | 7.6<br>7.6 | 10 | 5 | −50% | 7.6<br>7.4 | 10 | 10 | 0% | 7.6<br>7.6 |
| Sixth week | 10 | 45 | 350% | 7.3<br>7.4 | 10 | 12 | 20% | 7.4<br>7.4 | 5 | 4 | −20% | 7.8<br>7.6 | 10 | 10 | 0% | 7.3<br>7.2 |

In H. C. M. the animals differed very little from each other as far as external appearance is concerned. They were, for the most part, monopodal or bipodal. Sometimes, however, those with the chilomonad diet exhibited the branched form characteristic of the amoebae in W. C. M. with chilomonads. Those amoebae with the paramecium diet were usually slightly larger than the ones feeding on chilomonads, and during the last two weeks one specimen grew to be the largest amoeba in any of the cultures. Very large vacuoles were apparent in the amoebae in H. C. M. with the paramecium diet, and some individuals were spherical. These last two characteristics are probably associated with degeneration.

In Wilson's description (1900) of this giant amoeba, he mentions minute, elongate, and fusiform crystals in the endoplasm. Wilber (1942) describes the crystals in more detail and says that there are two types: (1) plate-like crystals, and (2) bipyramidal crystals, which are the more numerous. He states that these crystals (presumably both kinds) are formed from food in the food vacuoles. Wilson also mentions spherical bodies 8 $\mu$ in diameter and smaller, which resemble oil drops. Wilber gives the size range of these bodies as 2.5–8 $\mu$ in diameter, and

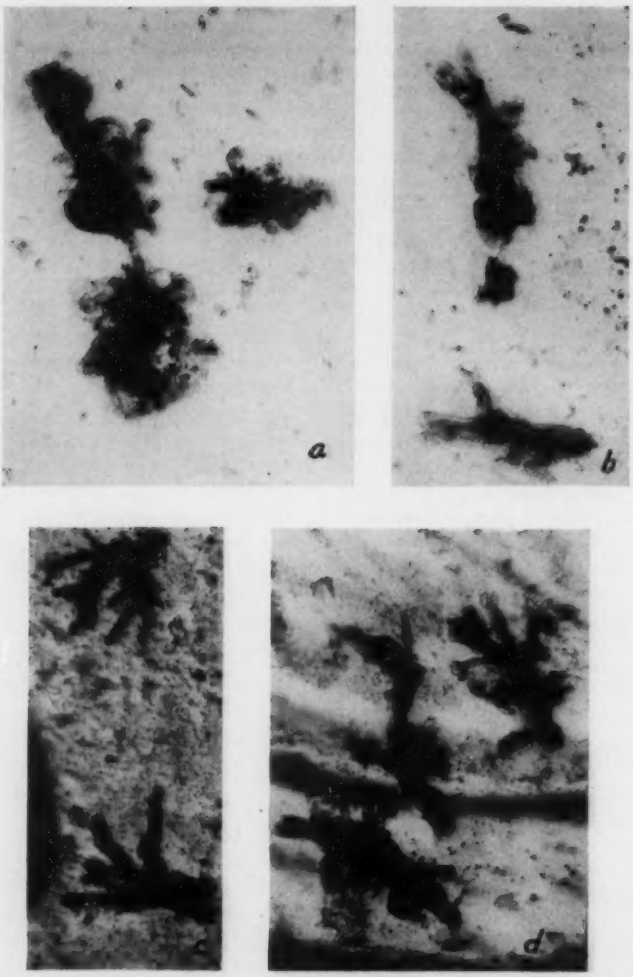


FIGURE 1. Photomicrographs of amoebae grown in wheat culture medium. a and b, those with paramecia as food organisms. c and d, those with chilomonads as food organisms.

concludes (1945) that they are formed from the crystals and the vacuole refractive bodies. He then says that the refractive spherical bodies function as reserve food in the amoeba.

While examining the stock and experimental amoebae under the compound microscope, differences in regard to the bipyramidal crystals and spherical bodies were noted. The plate-like crystals were so few in number that it was difficult to make a valid comparison in regard to their relative numbers and sizes.

In the stock cultures the amoebae had few spherical bodies, the largest of which were about 5 $\mu$ in diameter. The largest bipyramidal crystals were about 2.4 $\mu$ long.

The amoebae grown in W. C. M. with a diet of paramecia showed some larger bipyramidal crystals 2.8 $\mu$ long and a great number of smaller ones. There were few spherical bodies and the largest were only 3.1 $\mu$ in diameter.

Those specimens grown in W. C. M. and fed on chilomonads showed practically the same condition in regard to the bipyramidal crystals. However, there were very many large spherical bodies measuring 8.5 $\mu$ in diameter.

The spherical bodies of the amoebae grown in H. C. M. and fed paramecia were slightly more numerous and a little larger than the ones in the amoebae grown in W. C. M. with a paramecium diet. There were a few larger bipyramidal crystals 4.2 $\mu$ long, more 2.8 $\mu$ long and some smaller ones.

The amoebae grown in H. C. M. with chilomonads showed many large bipyramidal crystals 4.2 $\mu$ long, and the spherical bodies were very large and numerous. Many of the latter were 9.8 $\mu$ in diameter, and some were slightly flattened on one side so that they looked like a three-quarter-full moon.

## Discussion

With the exception of size and number of nuclei, *Amoeba proteus* and *A. carolinensis* are very much alike. Schaeffer (1926) states, "My study of this matter (a comparison of *A. proteus* and *A. carolinensis*) leads me to include *diffluens* (*proteus*) in the same genus with *Chaos* (the giant amoeba). These two species, as a matter of fact, resemble each other more closely than most other species within one genus."

From Wilber's (1942) description of the cytology of *A. carolinensis* it is seen that the giant amoeba resembles *A. proteus* very closely in regard to the shape and structure of the nuclei and the cytoplasmic inclusions. Wilber (1945) has also shown that these two forms are very much alike in respect to the function of the nuclei, the formation of the contractile vacuoles, and the formation and function of the spherical bodies.

When the data of these experiments are compared with similar work on *A. proteus*, it is shown that these two amoebae resemble each other in nutritional requirements. The numbers and relative sizes of the crystals and spherical bodies in the amoebae in my experiments agree in general with the results of similar studies by Mast (1939) and Mast and Hahnert (1935) on *A. proteus*. Concerning the numerous spherical bodies in the chilomonad-fed amoebae, Mast and Hahnert say, "This is of considerable importance for, as stated above, it indicates that the neutral red staining droplets found in abundance in *Chilomonas* but not in *Colpidium* function in the formation of the spherical bodies in *Amoeba*." My experiments indicate that this is also the case in *A. carolinensis*.

In examining the data of these nutrition studies it is seen that in general the amoebae with the most numerous and largest crystals and spherical bodies had the lowest reproduction rates. In view of the fact that the spherical bodies serve as reserve food (Wilber, 1945), these experiments indicate that even though chilomonads may be ingested and digested by the amoebae, this diet is not adequate for normal reproduction. The reproduction rates in the different cultures agree with the results of Reynolds (1938) and Williamson (1944) for *A. carolinensis*, but do not correspond with Williamson's data on *A. proteus*.

The relative sizes of the amoebae in the various cultures do not agree very closely with the observations during similar experiments by Mast (1939) on *A. proteus* and Williamson (1944) on *A. proteus* and *A. carolinensis*. In my experiments there was no increase in the size of the amoebae which had eaten ciliates, as found by both these authors, nor did the chilomonad-fed amoebae decrease in size, as found by Williamson.

In regard to the form of *A. carolinensis* while utilizing paramecia as food organisms, the results are in accord with those of Mast and Root (1916) for *A. proteus* and Williamson (1944) for *A. proteus* and *A. carolinensis*.

## NUCLEAR DIVISION

### Materials and Methods

Specimens were taken from the stock cultures and fixed with Carnoy's, Belling's, and Bouin's fixatives. Those fixed with Carnoy's and Belling's fluids were stained with Delafield's and Heidenhain's haematoxylin. Those fixed in Bouin's fixative were stained with Heidenhain's haematoxylin. Some specimens were sectioned at 10 $\mu$. Since those fixed with Carnoy's fixative and stained with Heidenhain's haematoxylin showed the nuclear structure best and stained the cytoplasmic inclusions very little, all the remaining work on nuclear divisions was done with these reagents.

In order to secure nuclear divisions, slides were made at various hours of the day. All the specimens showing nuclear division stages were fixed between 5:45 p.m. and 11:00 p.m. and were more or less spherical in form. The experiments are not extensive enough to warrant any statement as to a periodicity of mitosis. This seeming periodicity was probably a result of better selection toward the end of the experiment.

### Observations and Descriptions

*Resting nucleus*

The resting nucleus of *A. carolinensis* (Fig. 2a) is disc-shaped, and measures about 24 $\times$ 10 $\mu$. Immediately beneath a distinct nuclear membrane and adhering closely to it are darkly staining granules (peripheral granules), the largest of which are about 2 $\mu$ in diameter. The interior of the nucleus has a finely granular appearance and stains more lightly than the peripheral granules.

Schaeffer (1938) states that all the nuclei of an individual divide at the same time. Yet, nuclei at slightly different stages of division can be found in the same animal.

*Prophase*

In early prophase the nucleus enlarges apparently by a pulling away of the membrane from the central granules leaving a large endosome about 19 $\mu$ in greatest diameter. The nucleus now measures 27 $\times$ 15.4 $\mu$. Most of the peripheral granules are still rather small and stain more darkly than the endosome. Some of them however are slightly larger and less deeply stained than those in the resting nucleus. Faint strands running from the endosome to the periphery are now apparent.

In a little later stage (Fig. 2b and c) the nucleus has become more spherical measuring about 27 $\times$ 22 $\mu$. Some of the peripheral granules have become loosened

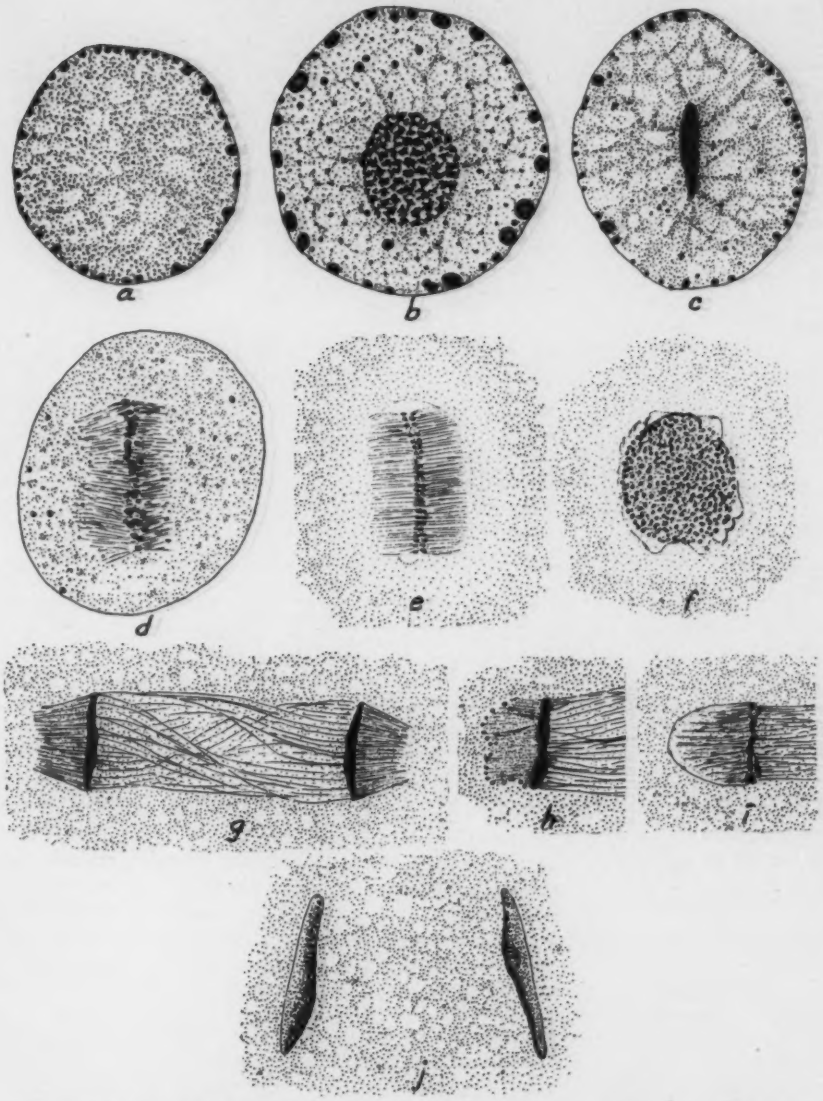


FIGURE 2. Camera lucida drawings of the stages of mitosis in *Amoeba carolinensis*. a. resting nucleus; b. prophase, face view; c. prophase, edge view; d. very late prophase showing chromosomes and spindle forming; e. metaphase, edge view; f. metaphase, polar view; g. late anaphase; h. early telophase, showing one plate with granules at the pole; i. a little later stage showing one pole with a delicate membrane and granules; j. late telophase with membrane fully formed. All drawings × 1660.

from the membrane, are more spherical, and show a lighter area in the center. The endosome has become smaller and more compact, and now stains about as deeply as the peripheral granules. At this stage the endosome is a thin disc measuring 9.3 $\mu$ in diameter and 1.5 $\mu$ in thickness. The reticulum connecting the endosome with the periphery is more evident now.

Very late prophase (Fig. 2d) shows the chromosomes becoming arranged on the plate which is 14.2 $\mu$ in diameter. Spindle fibers are distinct and the peripheral granules have practically disappeared. A lighter area in the cytoplasm immediately surrounding the nucleus is visible. The nucleus shown in Figure 2d was the only nucleus in the animal in this condition. The remaining nuclei were in metaphase or early anaphase.

*Metaphase*

At metaphase (Fig. 2e and f) the nuclear membrane has disappeared. There are, however, around the periphery of the plate delicate, blister-like structures which may be remnants of the nuclear membrane. The chromosomes, which are spherical or ellipsoidal, and perhaps about 300 in number, are arranged on a discoidal plate 13.2 $\mu$ in diameter. The split halves of the chromosomes in some cases can be seen. The spindle fibers are at right angles to the chromosome plate and are about 4 $\mu$ long. No centrioles or granules are apparent at the ends of the spindle fibers. There is still a lighter area in the cytoplasm around the figure.

*Anaphase*

During anaphase (Fig. 2g) the chromosome plate splits into two daughter plates which diminish in diameter with the chromosomes becoming so closely aggregated that they no longer can be distinguished individually. In late anaphase the plates measure 8.4 $\mu$ in diameter and are, for the most part, flat. A few are slightly arched or saucer-shaped. The spindle between the chromosome groups has in most cases become twisted as if both plates had rotated in opposite directions. The polar fibers seem to be finer and more numerous than the interzonal ones, and the polar areas appear slightly darker than the surrounding cytoplasm. The polar fibers have not shortened during anaphase but are in most cases 5 $\mu$ long, and the outside ones are inclined at an angle of about 60° to the plates.

In an amoeba containing thirty-three nuclei, twenty-nine are in late anaphase and four are in early telophase. The nuclei in anaphase have their polar fibers inclined at an angle of 60° to the chromosome plates. The four in early telophase have granules arranged on a more or less hemispherical surface or membrane (Fig. 2h and i). Twenty-nine of the spindles lie so that the distance between daughter chromosome groups can readily be measured. The average distance between groups is 34 $\mu$. The shortest distance is 10 $\mu$, and the longest distance is 62 $\mu$. Some of the chromosome plates are tilted at angles to each other so that one of a pair is seen in edge view, while the other is seen in polar view. Some pairs of plates, both showing in edge view, are twisted at angles of 30° to 150° to each other. In the twenty-one anaphase spindles which lend themselves to analysis, twenty have the spindle between the chromosome groups twisted clockwise, or to the apparent right (Fig. 2g). One spindle shows no twisting. This constancy in direction indicates that the twisting is caused not by external forces in the cytoplasm, but by forces inherent in the spindle apparatus.

*Telophase*

In early telophase (Fig. 2h) granules appear at the distal ends of the polar fibers. A delicate, more or less hemispherical, membrane forms at the poles and the ends of the fibers nearest the poles disappear (Fig. 2i).

Later telophase (Fig. 2j) shows the plates larger in diameter, less dense and more finely granular, with the membrane more flattened. The granules are more densely packed on the median sides of the nuclei where the membrane is scarcely visible except near the edge of the disc. The spindle fibers have completely disappeared by this time.

## Discussion

Mitosis in *A. carolinensis* as herein described is very similar to that of *A. proteus* as described by Chalkley and Daniel (1933), Chalkley (1936), and Liesche (1938). The figures and descriptions of the nuclear division stages of *A. proteus* agree with those of *A. carolinensis* with the following exceptions:

1. The nuclei and mitotic figures of *A. carolinensis* are approximately half the size of those of *A. proteus*. Also the chromosome number of *A. carolinensis* (probably near 300) seems to be about half that given by Liesche (500–600) for *A. proteus*.
2. No "spireme" (Liesche) stage was observed in *A. carolinensis*.
3. No granules (Chalkley and Daniel) were visible at the distal extremities of the spindle fibers at metaphase.
4. During metaphase and anaphase grouping of the distal ends of the polar spindle fibers is not so pronounced in *A. carolinensis*, and during anaphase the chromosome plates are only slightly arched.

## Summary

1. It is concluded that the giant amoeba described by Wilson (1900) is not a *Pelomyxa* but belongs to the genus *Amoeba* and should be designated *A. carolinensis*. *Chaos chaos* is considered to be invalid as a name, owing to the fact that it is based on Roesel's inadequate description.

2. Nutritional studies indicate that *A. carolinensis* has food requirements similar to those of *A. proteus* and that these two species react in a similar manner to the same type of food.

3. Nuclear division is described for *A. carolinensis*. The stages are similar to those described for *A. proteus*, and except for smaller size, correlated with a smaller number of chromosomes, the differences are insignificant.

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# THE ELIMINATION OF CHROMOSOMES IN THE MEIOTIC DIVISIONS OF BRACHYSTETHUS RUBRO-MACULATUS DALLAS

FRANZ SCHRADER

*Department of Zoology, Columbia University, New York*

The occurrence of meiotic abnormalities is anything but rare. However, in almost every instance it is the result of mechanical or physiological accidents and therefore sporadic and irregular. Hence, if in a certain species a departure from the normal process always takes a very particular form and is restricted to a certain region of the gonad, it is likely that it is not entirely accidental. If, furthermore, every male or female in the species is affected, it is safe to assume that we are dealing with a basic and well regulated condition, unorthodox though it may be.

The case in question is that of *Brachystethus rubromaculatus*, one of the pentatomid Hemiptera. It holds for all the males of the species. The testis here is composed of four lobes and in the fourth of these (counting from the side where the sperm duct makes its exit) the meiosis follows an abnormal but very definite course. In a way, this is analogous to the case of Loxa (Schrader, 1945a and b), but that is only in the sense that in both the exceptional development takes place in a certain lobe of every testis. The nature of the abnormality is quite different in the two species, as will appear in the account below. My chief interest lies in the possibility that such irregularities may be of use in the analysis of the ordinary mechanism of mitosis and in this as well as several succeeding studies I hope to show that they may throw some light on certain puzzling aspects of the division cycle.

## Material and Methods

The gonads of four males and one female were used in the investigation. The insects were collected near Turrialba, Costa Rica, during April and May, 1944. The material was fixed in Sanfelice and sectioned at 5 to 10 $\mu$. Gentian violet, haematoxylin and the Feulgen reaction were used in staining, the haematoxylin being especially useful in the analysis of spindle conditions whereas the Feulgen reaction is indispensable in following the maneuvers of the chromosomes. The main manifestations of the mitosis are so clear that they are well shown by pen and ink drawings—a method which in most other cases does not give a just presentation of spindle conditions.

I take pleasure in expressing my gratitude to Dr. E. N. Bressman and Mr. R. A. Nichols of the Inter-American Institute of Agricultural Sciences for facilitating the work. My special thanks are due to Dr. T. J. Grant of the U. S. Department of Agriculture, whose ready helpfulness did so much to further my researches in Costa Rica.

## Normal Spermatogenesis

The testis has only four lobes of which the first three show an orthodox spermatogenesis differing in no essential from that described in so many other pentatomids.

The fourth or "harlequin" lobe has about the same proportions as the rest and does not differ in any discernible way in its general organization.

The size differences among the chromosomes as seen in the spermatogonia are not very great. But it is possible to recognize one large, four medium and one small pair of autosomes. The X is a trifle larger than one of the small autosomes while the Y is the smallest chromosome of the complement (Fig. 1). Identification of the sex chromosomes was checked by examination of oogonial metaphases in the female.

The normal meiosis shows the usual prophase conditions, with the leptotene, synaptotene, pachytene, and diplotene stages followed by the confused period in which chromosomal behavior is difficult to analyze. This is succeeded by a diakinesis marked by the appearance of beautiful tetrads which, as they condense, are distributed around the nuclear periphery. Just before the breakdown of the nuclear membrane this rather even distribution begins to disappear and the chromosomes may even come in contact with each other in small groups of two or three each (Fig. 2). This collocation reaches its height when the membrane disintegrates. All of the chromosomes then huddle together in the middle of the nuclear space only to separate again almost at once to form the equatorial plate. In the latter they follow the characteristic pentatomid arrangement of the first metaphase, with one or both sex chromosomes taking a central position within a ring of autosomal tetrads (Fig. 3).

The sex chromosomes are marked by their heteropycnosis from the leptotene period on. Until the synaptic period they are well separated from each other, but then tend to come together. During the confused period they form a single, rounded chromatin nucleolus and it is not until after the early diakinesis that they once more become independent of each other. They divide equationally in the first, and segregate in the second division, so that the spermatids carry 6 + X or 6 + Y chromosomes (Figs. 3–5).

There is no interkinesis and the two centrioles at each pole have already separated to assume their position for the second division before the chromosomes have completed the anaphase movement of the first. This precocious behavior of the centrioles is however also encountered in several other pentatomids and does not interfere with the normal meiotic distribution of the chromosomes except in such extreme cases as Peromatus (Schrader, 1941b).

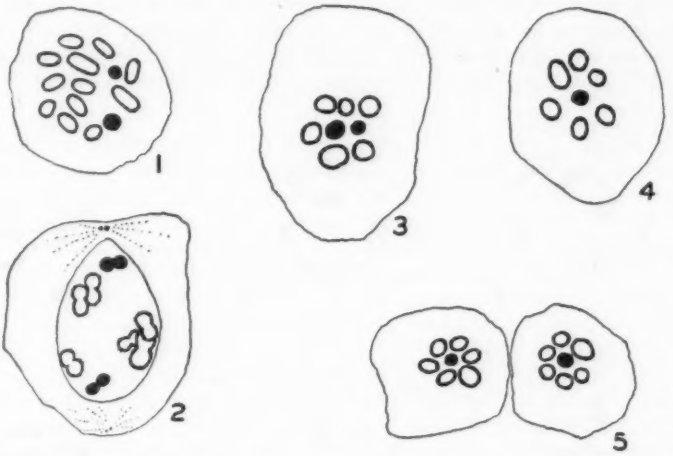
## Abnormal Spermatogenesis

### *First division*

Neither in the spermatogonial nor the meiotic behavior up to the late diakinesis does the harlequin lobe differ from the other three. Six autosomal tetrads are formed which resemble in every way those seen in the normal lobes (Fig. 6). Again, as these tetrads condense into the characteristic dumbbell shaped bodies, they and the sex chromosomes are well distributed around the nuclear periphery.

But then arises a difference in behavior from the normal. Here, too, the last phase prior to membrane disintegration witnesses a coming together of the chromosomes. In this fourth lobe, however, the collocation is both more regular and more pronounced. In nearly all cells the six tetrads form a single chain whose constituents maintain contact with the nuclear periphery. This chain is always more

or less equatorially placed (Fig. 7 and 8). The configuration is evidently the resultant of several different forces. Just as in normal cells, the tendency to collocate begins to manifest itself in late diakinesis. But in this fourth lobe the autosomal tetrads are also repelled by the two opposite poles at this early stage. Since the chromosomes are still confined within the nuclear membrane they consequently move into the middle region. The combination of polar repulsion, the tendency to collocate, and adhesion to the still intact nuclear membrane must perforce result in the formation of an equatorial chain.




Drawings were made from haematoxylin preparations, except for Figures 12 and 24. All figures magnified approximately 1400 ×. Autosomes drawn in outline, and sex chromosomes in solid black throughout.

FIGURE A. Normal spermatogenesis. 1. Spermatogonial metaphase; 12 autosomes and X (large) and Y. 2. Late diakinesis; beginning of autosomal clumping. 3. Metaphase of Division I; X and Y in middle. 4. Metaphase of Division II; X superimposed on Y. 5. Telophases of Division II showing two types of spermatids: 6 autosomes + Y, 6 autosomes + X.

As one might expect, there is no constant seriation in such a chain. When the nuclear membrane disintegrates, the chain is converted into an irregular clump just like the clump formed by the tetrads of normal cells that have undergone no such maneuvers (Fig. 9 and 10). Much less frequently is there a formation of two smaller aggregates instead of the single large one. The two sex chromosomes are included in such groupings only by accident and sooner or later they always separate from the autosomes and assume an independent position. This is not necessarily an equatorial one at first (Fig. 8–10).

It is, however, in the establishment of the first metaphase that the most striking departures from an orthodox behavior are evinced. As already stated, the breakdown of the nuclear membrane is followed immediately by the clumping of the autosomal tetrads in the midregion. In many cells this may, however, be halted temporarily if a central core of spindle fibers is formed quickly between the poles. The clumping autosomes may then be applied to these central fibers in a half moon

configuration for a moment (Fig. 11). But in any case, before the metaphase spindle has assumed final shape, the autosomal aggregate is shifted out of this general middle region. Almost always this movement seems to be a sudden one and frequently the aggregate comes to rest rather close to the lateral wall of the cell, the direction of the shift being toward the side and never toward the poles. The cell frequently bulges out on the side on which the aggregate is located (Fig. 14). If instead of one aggregate, two smaller ones have been formed, they undergo similar reactions and often become displaced toward opposite sides of the cell (Fig. 13).

The beginning of this autosomal shift sees the X and Y in no definite position although usually in the vicinity of the polar axis. Frequently they are situated at some distance from the equatorial plane and bear neither a constant positional relation to each other nor a mitotic orientation toward the centrioles (Fig. 9 and 10).

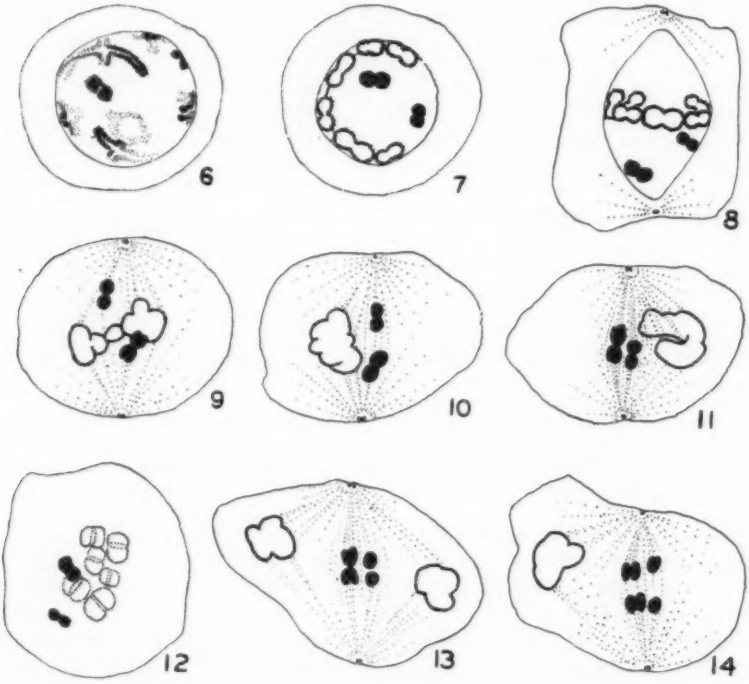



FIGURE B. Abnormal spermatogenesis. Division I from diakinesis to early anaphase. 6. Mid diakinesis showing tetrads of normal appearance. 7. Late diakinesis in polar view; autosomal tetrads forming equatorial chain. 8. Late diakinesis in side view. 9. Early stage in clumping of autosomal tetrads. 10. Beginning of movement of autosomal aggregate away from polar axis. 11. Displaced autosomal aggregate applied to central portion of spindle in half moon form; X and Y assuming equatorial position. 12. Feulgen preparation corresponding to Figures 9 or 10 showing that tetrads retain their individuality in the aggregate. 13. Autosomes in two aggregates, both shunted out of polar axis. 14. Early anaphase, with X clearly showing "tertiary" split; characteristic displacement of autosomal aggregate.

However this situation is quickly altered and as the autosomal aggregate moves toward the side of the cell, the X and Y approach very close to the polar axis and assume a position in the equator with a definite orientation toward the poles (Fig. 11 and 13).

In all of the hundred or more cells observed at this stage, such a configuration of autosomes and sex chromosomes is always maintained. Since in the prometaphase the two types of chromosomes form one general group albeit not always in contact with each other, it is clear that the later separation is not an accidental one. The unusual shift of the autosomes is equivalent to an actual extrusion from the mid-region of the cell.

Despite their distance from the polar axis, the autosomes continue to be connected with both poles by chromosomal spindle fibers. This is at first glance rather surprising since with ordinary stains like gentian violet and haematoxylin the autosomal aggregate appears as a solid, structureless mass which plainly suggests degeneration. In good Feulgen preparations, however, it becomes clear that the autosomal tetrads have by no means lost their individuality. All six of them can be easily distinguished, lying in a substance which does not stain with Feulgen and thus reveals the individual chromosomes (Fig. 12). This substance, which with other stains becomes just as dark as the chromosomes themselves, resembles the material surrounding the sex chromosomes of certain reduviid Hemiptera (Troedsson, 1944) and may also be akin to the "flocculent, whey-like coagulum" which envelops some regions of the chromosomes in Olfersia (Cooper, 1944). In Brachystethus it is evidently formed when the chromosomes clump at prometaphase and it persists through both meiotic divisions.

In the middle of the cell, between the two poles, there is a well-formed spindle of normal length, which however is smaller in diameter than the spindle of normal cells. This accommodates the two sex chromosomes, which are located in the middle of the spindle substance just as they would be if the autosomes were free to form a ring around them.

Despite the presence of the chromosomal fibers which connect the autosomes with the poles, the autosomal aggregate behaves more or less like an inert mass in the mitosis that follows. While the sex chromosomes undergo an equational division and approach the poles, the autosomes near the periphery of the cell undergo no movement (Fig. 14–17). It is only when the dividing cell elongates and becomes narrow that the autosomal clump is moved toward the midline (Fig. 18). If it there comes in contact with the expanding interzonal region or "Stemmkörper" it may be swept along into one of the two daughter cells, but very often it is not until the cleavage constriction is being completed that the aggregate is definitely included in either of the resulting cells (Fig. 19 and 20). Apparently the chromosomal fibers exert little or no traction at this time. So far as one can tell, the inclusion of the autosomes in either daughter cell occurs entirely at random.

It is of interest to note that although in its essentials the equational division of the sex chromosomes is always accomplished successfully, there are certain features that distinguish it from the corresponding process in normal cells. In the first place, the sex chromosomes and especially the X clearly show a tertiary split already at this first metaphase—a split which is not utilized until the first somatic division in the egg (Fig. 13–15). This is sometimes indicated in normal cells also, but never so strikingly as here where it appears after all three of the stains used. The second

feature lies in the anaphase behavior of the sex chromosomes. Although both start out from an equatorial position, the early anaphase often shows the chromatids of the Y much closer to the poles than those of the X (Fig. 15). In other words, the Y precedes the X in the poleward movement. When there is such a disparity of movement, there is frequently a shift during mid anaphase through which these four chromatids are all brought into the exact polar axis in a single line. Since as just stated, the chromatids of the Y move more quickly, they constitute the extremities in this tandem arrangement, whereas the two X chromatids are in between (Fig. 16). Such a seriation strongly resembles that which characterizes the second di-

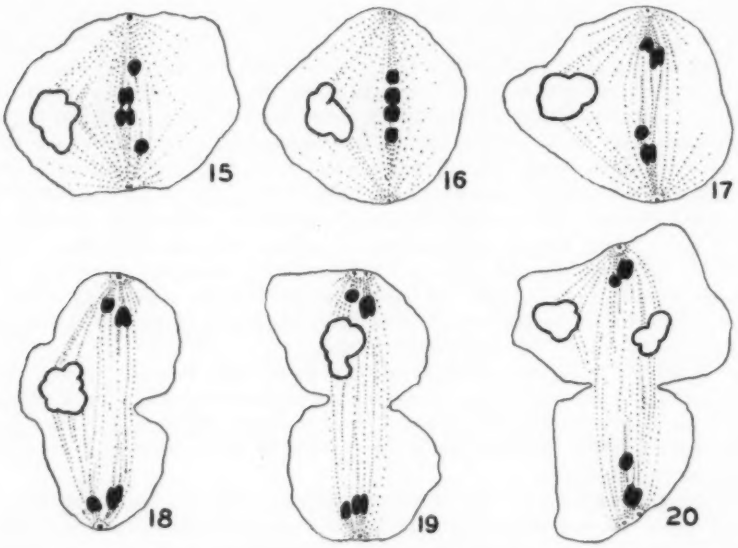



FIGURE C. Abnormal spermatogenesis. Division I, anaphase and telophase. 15. Early anaphase with Y preceding to the poles. 16. Early anaphase; X chromatids have moved into polar axis between separating Y chromatids. 17. Mid anaphase. 18. Late anaphase; autosomal aggregate returning to axial region. 19. Early telophase; an X and a Y at each pole with the autosomal aggregate included in upper cell. 20. Late telophase with two autosomal aggregates both included in upper cell.

vision of the coccid Protortonia (Schrader, 1931), and there is little doubt that similar forces are involved. This anaphasic shift does not affect the result of the division; one X and one Y chromatid go into each of the resulting second spermatocytes.

Thus in the majority of cases, two types of second spermatocytes are produced: one carries an X and a Y, as well as the clumped autosomes; the other contains only the two sex chromosomes (Fig. 19). If the autosomes have been aggregated in two masses before metaphase, these may both go to the same pole or to opposite poles, apparently at random (Fig. 20). In no case is there a division of the individual tetrads.

*Second division*

As in normal cells, the centriole at each pole of the first spindle is divided and the two daughter centrioles separate some time before the division has been finished (Fig. 21). Each moves through an arc of 90° and the new polar axis for the second division is therefore at right angles to the first. Even while still in telophase, the two sex chromosomes often respond to the new poles and move toward them (Fig. 21). However, this precocious movement is soon reversed and the X and Y then come together in the middle of the new spindle in their orthodox "touch and go" pairing (Fig. 22 and 28) and it is only following this that they separate in the regular segregation toward opposite poles. There is no indication of any interkinesis.

The autosomal aggregate may at first remain in the general region where it has entered the cell upon completion of the first division, but in other cases it approaches closely to the two sex chromosomes that are near the new polar axis (Fig. 21). Such an approach is only temporary, however, and before the second division is initiated the aggregate is always extruded from this middle region just as it was in the first division (Fig. 23). The configuration of the second division thus almost duplicates that of the first.

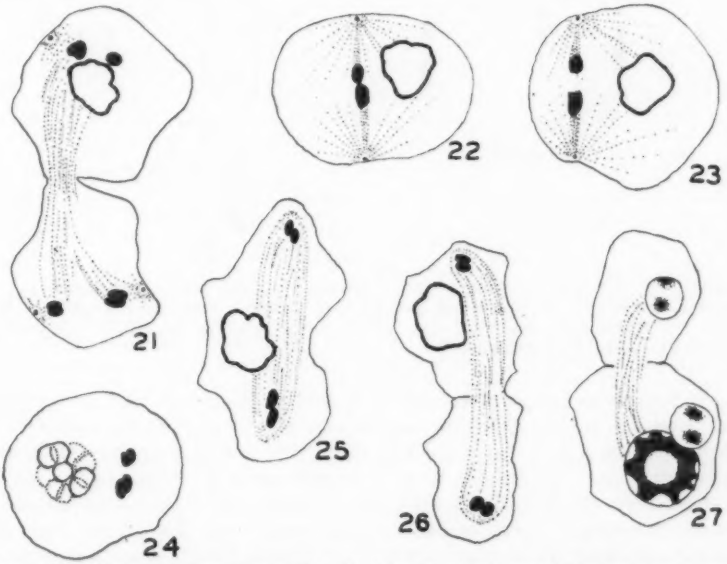



FIGURE D. Abnormal spermatogenesis. Division II of cell containing autosomal aggregate (all drawings except Fig. 21 show X below Y). 21. Beginning of Division II; X and Y reacting to new position of poles. 22. Touch and go pairing of X and Y; autosomal aggregate shunted away from polar axis. 23. Early anaphase; X and Y going to opposite poles; autosomal aggregate in characteristic displacement. 24. Feulgen preparation showing that tetrads still appear unaltered in early phase of Division II. 25. Anaphase; autosomal aggregate returning toward polar axis. 26. Telophase; autosomal aggregate included in cell with Y. 27. Spermatids; upper cell carries only a micronucleus with the Y half chromatids; lower cell with micronucleus containing X half chromatids and autosomal nucleus.

The autosomal tetrads are still intact and are still imbedded in the substance that stains intensely with gentian violet and haematoxylin. However, the aggregate is usually smaller than it was just prior to the first division, a fact that results from a greater crowding of the tetrads, as can be seen in Feulgen preparations (compare Fig. 24 with Fig. 12). Again, chromosomal fibers are formed (Fig. 22), although the aggregate seems to be moved about as a more or less passive body (Fig. 25 and 26). In short, just as in the first division it is only the sex chromosomes that are involved in the regular mitotic mechanism. But now, as in normal second divisions, the process is reductional and the resulting cells receive only an X or a Y (Fig. 26 and 30). The autosomes if present are once more included in either cell as a group, and so far as one can tell, on the basis of chance (Fig. 23–26).

The spermatids that result from these two peculiar divisions thus either carry only one sex chromosome (the X or the Y) or else they contain the autosomal aggregate in addition. In other words, there are four main types of spermatids: X; Y; X + autosomes; Y+ autosomes (Fig. 26 and 30).

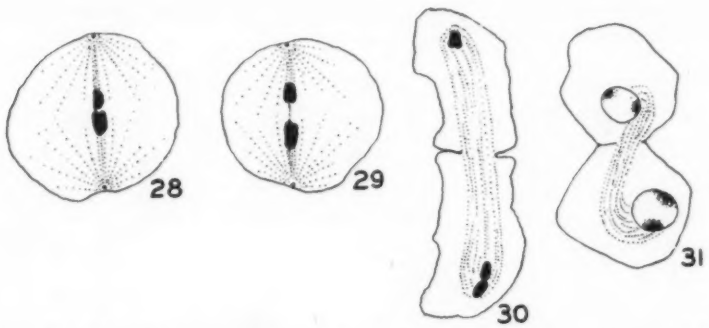



FIGURE E. Abnormal spermatogenesis. Division II of cell lacking autosomes (all drawings show X below Y). 28. Touch and go pairing of X and Y. 29. Early anaphase. 30. Late telophase. 31. Spermatids; upper cell with micronucleus containing the two Y half chromatids, lower with two X half chromatids.

If the autosomes are gathered in two instead of the usual single group prior to the first division, the resulting spermatids may of course carry intermediate numbers of chromosomes. But for the great majority of cells they are transmitted in a single aggregate. Throughout there is no attempt at a division of the individual autosomal tetrad, and it is as an aggregate of tetrads that the autosomes enter the spermatid. Obviously there is some factor which interferes with their mitotic mechanism—a factor which interferes in no way with that of the sex chromosomes. The latter behave just as they do in the normal cells of neighboring lobes of the testis.

*Spermateleosis*

The first steps after the telophase of the second division parallel the normal course of events. The transformation of the autosomal mass into the spherical nuclear structure shown in Figure 27 is peculiar, though in later stages it approaches

closely the condition seen in the normal spermatid. In these abnormal cells however, the sex chromosome at first forms a micronucleus which is distinct from the larger autosomal nucleus (Fig. 27). But sooner or later, the micronucleus becomes applied to the large nucleus and gradually merges with it.

When, as in most cases, a single sex chromosome constitutes the only chromosomal constituent of the spermatid, its behavior differs in no way from that in cells containing the autosomes as well. In either case a tiny nucleus is formed by each sex chromosome and in this the constituent half chromatids separate to form two distinct chromosomal bodies. (Fig. 27 and 31). This precocious separation is not surprising when it is remembered that already in the first metaphase these half chromatids can be clearly identified as such.

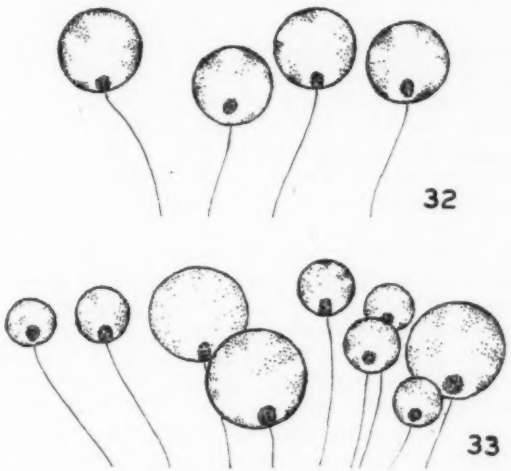



FIGURE F. Spermatids before elongation. 32. Normal spermatids shown to indicate size. 33. Abnormal spermatids at stage corresponding to Figure 32. Largest spermatids contain all autosomes as well as either X or Y. Smaller spermatids carry either X or Y; latter is the smallest.

The process of transformation into the sperm seems at first alike in all spermatids and homologous to that observed in the normal lobes. This is true whether they carry only a single chromosome or the autosomes as well. As might be expected, the former are distinctly smaller than the corresponding normal cells and nuclei (Fig. 32 and 33); the latter on the other hand are markedly larger. It is also interesting to note that it is often possible to distinguish between the small sperms carrying either an X or a Y. The latter are the smaller of the two (Fig. 33). However, when the rounded sperm heads begin to elongate, those which carry only a sex chromosome begin to stain less intensely than either normal or "autosomal" abnormal sperms, and as the elongation into the typical tenuous sperm head continues it becomes more and more difficult to trace their further fate even in Feulgen preparations. It is likely that the "sex chromosome sperms" never attain

the final stages of sperm formation. However, this cannot be made certain without smear preparations for even in very thick sections the long sperm heads are practically always cut and it is impossible to decide whether one is dealing with an abnormally small sperm or only a portion of a larger sperm. The fate of the giant sperms, however, is subject to no such doubt. They continue their development to the fully formed stage, enter the sperm duct, and are there mingled with the normal sperms from the normal lobes.

## General Considerations

The cytological features that characterize this abnormal development are thus of the most striking sort. The most obvious one lies in the clumping of the autosomes. A temporary collocation of chromosomes is of course encountered as a normal occurrence in many species, but is there, confined—as it is in the normal lobes of Brachystethus—to a very brief, almost momentary period immediately after the breakdown of the nuclear membrane in prometaphase. In Brachystethus there is some tendency toward a collocation of autosomes even before this event. But in the abnormal lobe, the clump that is finally formed is not resolved again, and this condition is maintained through both meiotic divisions and into sperm formation. Moreover this collocation of chromosomes is so intimate that only a Feulgen preparation reveals that there has not been an actual fusion.

Although the further behavior of this autosomal aggregate suggests strongly that it is shunted about more or less like a passive body during cell division, it nevertheless evinces certain reactions which indicate that it is not completely inert. In diakinesis, these autosomal tetrads duplicate the behavior of normal tetrads in establishing contact with the nuclear periphery; toward the end of the diakinetic period they evince a reaction to the two poles in taking up an equatorial position in contact with the nuclear membrane; after the disappearance of the latter they form chromosomal fibers to the poles; and almost simultaneously they move out of the midregion of the cell with a suddenness that bespeaks a forcible displacement.

It is possible and even likely that this movement toward the side of the cell is due to the same centriolar repulsion which forces the chromosomes into the equatorial plane while they are still held within the confines of the nuclear membrane. An influence of centrioles on the chromosomes prior to the dissolution of the nuclear membrane is observed in certain other forms also (for instance in Anisolabis, Schrader, 1941a). But not often is it exerted so as to bring about an equatorial placement at so early a stage, even though a role in the later final formation of the equatorial plate is frequently assigned to it.

It will be realized, however, that even in normal cases, additional factors must function to restrict the chromosomes to the middle of the equatorial plane. Repulsion from the two poles alone cannot do that. It is possible that the chromosomes are thus confined to the midregion simply because of surface tension conditions that prevail in the spindle body at the time of metaphase. The escape of the autosomes in the present case might then be attributed to abnormalities in the spindle, say to untoward alterations in the timing of the normal viscosity changes. But that is clearly not the entire explanation since the autosomes are not accompanied in their displacement by the two sex chromosomes. The latter remain in the spindle in a quite orthodox position. Since the cytoplasm, the centriolar forces,

and the general spindle conditions are identical for both autosomes and sex chromosomes, it is therefore in the chromosomes themselves that the explanation must be sought. More specifically, the question to be solved is why the autosomes do not respond to the forces which at metaphase counteract the influence of the centers and confine the chromosomes to the middle of the equatorial plane.

The abnormal condition of the autosomes is not indicated by any striking behavior during the prophase. There is only a more pronounced tendency to assume an equatorial position at prometaphase and the formation of chains that are absent in normal cells. Also, after the aggregate has been formed, Feulgen preparations show the component tetrads to be somewhat swollen and less intensely stained than are the prometaphase tetrads. This probably indicates the first step in a return to a diffuse state. Such a regressive condition may in some way be responsible for the special maneuvers of the autosomes, for the X and Y chromosomes which during this time remain fully condensed behave just like the sex chromosomes of normal cells. But it is not certain that this would suffice as an explanation since in addition to the clumping of the autosomes one must also account for their elimination from the midregion and their failure to take the first steps in division. For it must be remembered that the initial separation of daughter chromosomes is an autonomous process which is independent of spindle action. Nevertheless, although the Brachystethus tetrads appear ready for such a step and the line of demarcation between the paired chromosomes is clearly marked and complete, no separation ever occurs. Either there is no mutual repulsion or else the pellicle and the achromatic constituents of the tetrads prevent the normal division. In any case it is clear that some of the basic reactions of the chromosomes have been altered.

It is natural to seek a parallel in other cases already on record. The investigation of certain echinoderm hybrids by Baltzer (1910) is especially pertinent. Baltzer found that in certain of these crosses, the paternal chromosomes are eliminated during cleavage. There, too, is a tendency for such chromosomes to clump, and there also a formation of chromosomal spindle fibers occurs nevertheless. Likewise there is sometimes a lateral elimination of these chromosomes although they usually simply lag in the middle of the spindle. However, all this does not occur until the anaphase is well advanced and the various configurations are by no means as constant as they are in Brachystethus. After a careful analysis, Baltzer concluded that it is the chromosomes themselves rather than the plasma that is responsible for the elimination. That such a conclusion is warranted for the case of Brachystethus, as well, has already been pointed out. The difference in behavior between the autosomes and the sex chromosomes in the same plasma makes it unavoidable.

The elimination of chromosomes consequent on a loss of their kinetochores is observed regularly in certain molluscs (Pollister, and Pollister, 1943). Such an explanation is worthy of serious consideration in the case of Brachystethus. However, there can be only a partial loss of the kinetochore activity because some chromosomal fibers are evidently still being formed. Since we are dealing here with a diffuse kinetochore, such a partial loss is easily conceivable, but it must be confessed that the akinetic chromosomes of molluscs present very different elimination pictures than those seen in Brachystethus.

The conditions in such coccids as Phenacoccus (Hughes-Schrader, 1935) may also be germane to the present case. There, too, certain chromosomes betray a

tendency to clump, and it is these chromosomes that become inert and degenerate within one or two succeeding divisions. Nevertheless, these chromosomes form normal chromosomal fibers.

But all these other instances are themselves in need of further explanation. The only conclusion that protrudes itself in such an analysis is that the abnormality is to be sought in the basic organization of these forms and is not a superficial and accidental one. The alterations that are involved primarily affect the chromosomes and influence their reactions to each other and to the mitotic mechanism.

## Evolutionary Aspects

It is very questionable whether spermatids carrying only an X or a Y chromosome ever develop into mature sperms. That, however, is not true of the giant sperms which in addition to a sex chromosome carry the full set of autosomes unaffected by any meiotic mitosis (i.e., four times the number of autosomes contained in a normal sperm). But though these large sperms enter the sperm duct and mingle with the normal sperms, it is doubtful whether they ever become functional in the hereditary sense. Of the seven specimens of Brachystethus that I have examined, none was marked by unusual morphological features such as confidently might be expected if a sperm nucleus with a quadruple set of autosomes joins the haploid nucleus of a normal egg.

But if these numerous giant sperms play no direct role in the hereditary mechanism of the species, it becomes a matter of wonder that such an extensive development of abnormal gametes could have withstood the effects of natural selection. For it must be remembered that the testis of Brachystethus has only four lobes and if the sperms of one of these do not function in the genetic determination of the embryo, we are confronted with a prodigious waste which is added to that which occurs normally in the reproduction of most male animals.

Such a waste is paralleled in several species of Loxa (Schrader, 1945a and b) where there is likewise an abnormal lobe in every testis. Although the nature of the aberrancy is different in the two cases, they are similar in that both encompass the production of a huge number of sperms which carry many times the normal number of chromosomes. It seems strange that two processes so diverse in their abnormalities should culminate in gametes whose general characters are so similar.

The explanation may lie in the fact that though these large sperms take no part in the direct control of the heredity of the species, they may nevertheless be important in its welfare. Since they are normal in every respect except size, it is more than likely that they enter the egg like the normal sperms. It also must be recalled that polyspermy is almost universal in the fertilization of insect eggs and that in many species the number of supernumerary sperms is very high. The entrance of genetically inert sperms would probably offer no difficulties in the regular fertilization process and a union between two normal pronuclei would proceed as usual.

It is clear from evidence in marine eggs that the breakdown of gamete nuclei releases substances which have a far reaching influence on the reactions in the egg. It is likely that such substances are present in abnormally large quantities in the giant sperms and that therefore their possibly beneficial effects are increased. Again, if the developing embryo utilizes the nucleo-proteins that are brought in by

supernumerary sperms, an advantage might well accrue to a species which has such substances available in the unusually large amounts that are represented in the giant sperms. In short, the latter may play a not inconsiderable role in the embryology.

If that is the case, the harlequin lobe would in no sense be a useless organ. The development of such a lobe in the testes of two genera that are taxonomically as diverse as Brachystèthus and Loxa may well have been due to a nongenetic but metabolic advantage that is thereby conferred on the developing embryos.

## Summary

1. The fourth lobe in all testes of the pentatomid *Brachystethus rubromaculatus* shows an aberrant meiosis of a very definite character.

2. The first indication of this aberrancy is found in a chain formation and clumping of the autosomal tetrads just prior to metaphase. The two sex chromosomes are not included in this aggregation of autosomes.

3. At metaphase the X and the Y take a normal position in the spindle but the aggregate of autosomes is shunted laterally out of the middle region of the cell, away from the polar axis.

4. The two sex chromosomes divide equationally in the first division while the autosomal aggregate passes unaltered to either pole.

5. In the second division the X and Y again behave as in a normal meiosis and separate to opposite poles. The autosomal aggregate, if present, passes to either pole apparently at random.

6. Since the clumped autosomal tetrads pass undivided through both divisions while the sex chromosomes behave normally, the following four main types of spermatids are produced: X; Y; X + autosomes; Y + autosomes.

7. The mechanics and evolution of so constant an aberrancy are discussed.

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# AN INVESTIGATION OF CROSS STRIATIONS AND MYOSIN FILAMENTS IN MUSCLE *

C. E. HALL, M. A. JAKUS, AND F. O. SCHMITT

*Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts*

## INTRODUCTION

Present information on muscle structure is of two rather distinct types, that derived from histological studies through direct observation with the light microscope, and that deduced from indirect methods such as involve polarized light microscopy, streaming double refraction of myosin solutions or x-ray diffraction. Histological studies have always been limited by the inadequate resolving power of light microscopes; the indirect methods have been limited by the fact that they can supply information only in proportion to the correctness of the necessary assumptions. Although valuable knowledge has resulted from both types of approach, there is a lack of definite information on structures below the limit of light microscope resolution. The electron microscope provides the possibility of extending direct observation into a range of dimensions reached hitherto only by indirect methods and conjecture. Since success in biological electron microscopy depends to a large extent on the development of techniques suitable for the material, these initial observations may be expanded as new techniques are developed.

It is impossible in the present space to summarize the previous research on muscle or the ideas put forth concerning its structure; reference to the literature will be limited to those papers which provide a basis for the present study. A general review has been presented by Fenn (1945) while the histological aspects have been reviewed by Jordan (1933). Polarized light investigations have been reviewed by Schmidt (1937) and by Weber (1934). Results of investigations of streaming double refraction in myosin solutions may be found in the papers of Edsall (1930, 1942), von Muralt and Edsall (1930), Mehl (1938) and Dainty, Kleinzeller, Lawrence, Miall, Needham, Needham and Shen (1944). Astbury and Dickinson (1940) and Astbury (1942) described wide-angle x-ray diagrams of muscle and myosin, and Bear (1944, 1945) described small-angle x-ray patterns of various muscles. A discussion of muscle physics has been presented in a review by Ramsey (1944).

In the past, the electron microscope has been used very little in the study of muscle structure. Richards, Anderson, and Hance (1942) have shown electron micrographs of a wedge-cut section of cockroach striated muscle in which each darker band can be seen to consist of three components. Sections of muscle tissue cut by another method were examined by Sjöstrand (1943) but the electron micrographs bear no obvious relation to known structures in muscle. In both these stud-

* The substance of this paper was presented at the meetings of the Electron Microscope Society of America on December 1, 1945.

ies the object was primarily to investigate sectioning techniques for electron microscopy rather than to study muscle itself.

Ardenne and Weber (1941) published electron micrographs of filamentous myosin particles, which are undoubtedly to be identified with the asymmetrical particles responsible for the streaming birefringence phenomena in myosin sols. Hall, Jakus, and Schmitt (1945) have studied another fibrous protein from molluscan muscles and correlated the structure with the x-ray diffraction results described by Bear (1944).

## Electron Microscope Observations of Myofibrils

### *Preparation of specimens*

A primary difficulty in the study of striated muscle with the electron microscope is the preparation of the tissue thin enough to be partially transparent to the electron beam. In view of the difficulties connected with present microtome techniques, a preparative method was sought in another direction. One procedure which has been used previously depends on the tendency of many materials to fragment along natural cleavage boundaries. If such tendency to natural cleavage exists, it may be assisted by chemical or physical means or both. Thus collagenous tissue may be separated into fine fibrils by mechanical "teasing" and the separation is facilitated by weak acid (Schmitt, Hall, and Jakus, 1942). Such methods of fragmentation are applicable to many materials and are readily adapted to striated muscle.

Fresh muscle is fixed in 10 per cent formalin, cut into small pieces and subjected to mechanical agitation in a Waring Blendor. When the resulting suspension is lightly centrifuged to throw down the larger fragments, the supernatant exhibits the characteristic sheen associated with fibrous suspensions. It consists mostly of thin fibrils which may be washed and applied directly to a conventional electron microscope specimen grid with collodion membrane. These fibrils are to be identified with the myofibrils, or sarcostyles, of the muscle fiber.

Most of the observations to be described were made on fibrils from the leg muscles of frog and rabbit. The muscles of lobster and scallop (striated portion) were found to contain relatively large fibrils which were usually quite opaque in the electron microscope. It was necessary to apply fixatives to all these muscles in order to obtain intact fibrils. Wing muscles of the fly, on the other hand, could be teased apart in weak saline solution without previous fixation.

Most myofibrils from frog muscle have widths between 0.5 and 1.0 $\mu$, although a few may be found as wide as 3.0 $\mu$ or as narrow as 0.2 $\mu$. They appear to be ribbon-shaped on the electron microscope specimen holder but it is not possible to say whether this shape results from forces produced during drying or whether it represents the form of the myofibril in the intact tissue.

Although most of the structural features of the myofibril can be observed without the use of stain, contrast in the image may be increased by the application of phosphotungstic acid to the specimen (0.1 per cent solution at pH 3–5 for about one minute). The staining procedure is found to be particularly useful in increasing the contrast of the myosin filaments.

An RCA electron microscope Type B with accelerating voltage raised to 65 KV was used throughout the study. A higher voltage would probably be advantageous for this type of material but is not readily obtainable from the standard power supply.

*Unstained fibrils*

Unstained myofibrils from striated muscle are characterized by a succession of transverse bands of varying density, the main features of which can readily be identified with the bands previously described in histological studies (Jordan, 1933). An electron micrograph of a typical fibril from a slightly stretched frog sartorius is shown in Figure 1, together with the histological designations. The repeating unit, or sarcomere, is bounded terminally by a narrow dense band usually referred to as the *Z* membrane or telophragma. This band is in the center of a light region, the *I*[s] (or *J*) band, so called because it is relatively isotropic. Contiguous with *I* is the sharply-defined *A* (or *Q*) band which is optically anisotropic and has a higher scattering power for the electron beam than does *I*. In some myofibrils the *H* disc (or median disc of Hensen) appears as a lighter region in the middle of the *A* band (Fig. 1). Bisecting the *A*, or the *H* disc if it is present, is the narrow dark *M* band (or mesophragma). There may also appear, in either half of the *I* band, a relatively dark band designated as *N* in Figure 2. These bands are not always present and have been considered by some cytologists to be artifacts resulting from the lining up of granules. In the present study they have been noted in rabbit muscles but are either faint or absent in frog muscles. The clarity of their appearance in rabbit fibrils leaves no doubt as to their existence in this formalin-fixed material.

Myofibrils from rabbit muscle usually show two to four fine cross bands symmetrically disposed with respect to the *M* band and near it. They are beyond light microscope resolution, being about 0.1 $\mu$ from center to center, and represent a periodic variation in the dense material of the *A* band.

In favorable cases fibrils can be seen to consist of longitudinal myosin filaments which are difficult to resolve in unstained fibrils except where they fray out at the edges. They are visible in various parts of Figures 1 and 2.

*Stained fibrils*

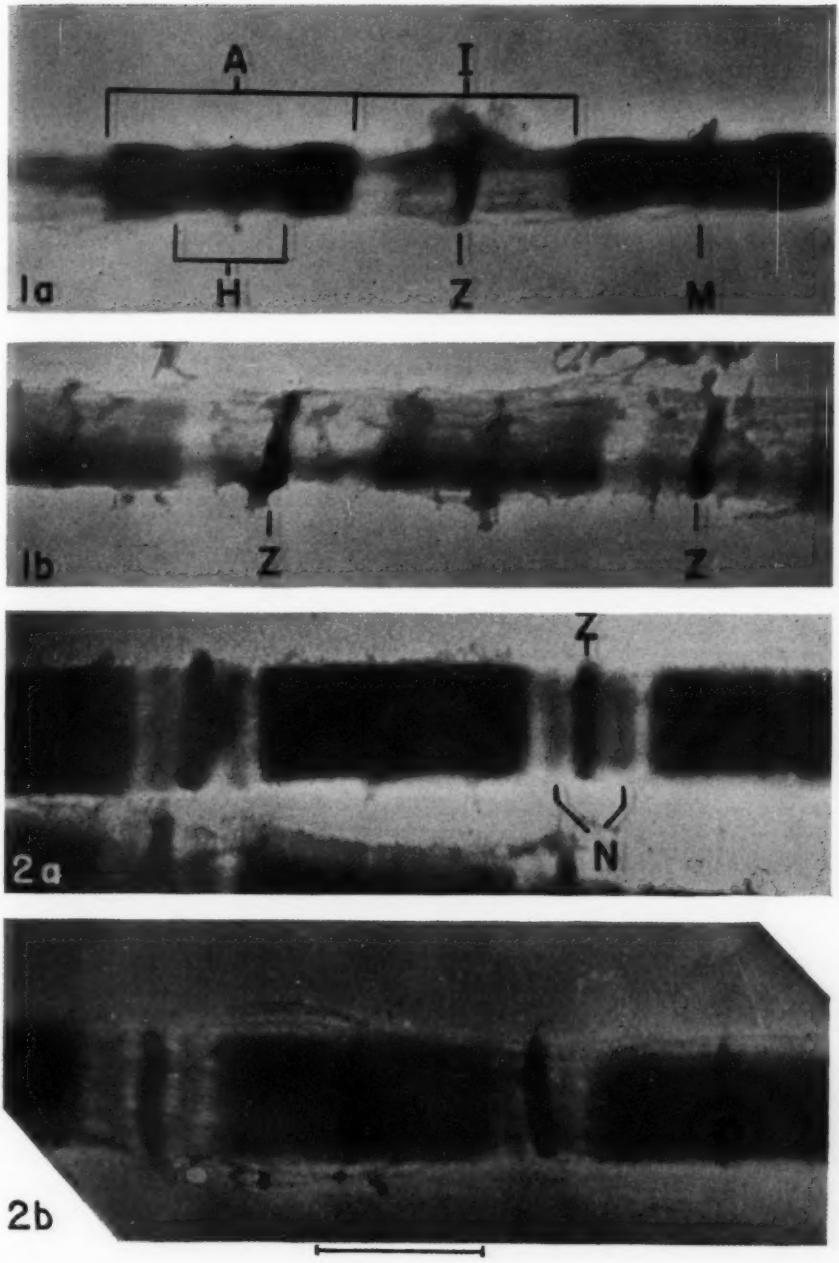
Phosphotungstic acid combines with the cross bands roughly in proportion to their intrinsic density and serves to increase the contrast of the structures described above. Segments of stretched myofibrils stained with phosphotungstic acid are shown in Figure 3. The *A* band is stained more heavily than is the *I* band, and the *M* and *Z* bands appear quite opaque. When the *H* disc is present, it absorbs less stain than does the remainder of *A*.

Besides accentuating the transverse bands, phosphotungstic acid enhances the contrast of the longitudinal myosin filaments. These range in width from 50 to 250 Å and extend continuously, and in relatively straight lines, through both *A* and *I* bands. Although the filaments are usually indistinguishable within the dense *Z* bands, they can be traced through several successive sarcomeres when the *Z* bands are partially disintegrated. In the *A* band the filaments are relatively dense, sharply defined, and almost parallel in orientation. In the *I* band they are less perfectly aligned and of lower density. The higher density of the *A* band appears to result from the higher density of the component filaments.

FIGURE 1. Myofibrils from frog sartorius, stretched about 30 per cent, unstained. × 25,000. All myofibrils shown in this and subsequent figures were fixed in 10 per cent formalin.

FIGURE 2. Myofibrils from rabbit leg muscle, unstained. × 25,000.

PLATE I



In both bands, the myosin filaments present a knotted or beaded appearance with frequent constrictions and variations in density. The nodes are often almost equidistant along the filament and, in some regions of the fibril, they may be aligned laterally to produce a fine cross striation with a period of about 400 Å in the direction of the fibril axis. However, this feature has not been found sufficiently reproducible or regular to be designated as a periodic spacing in the nature of those found in collagen and in clam muscle fibrils.

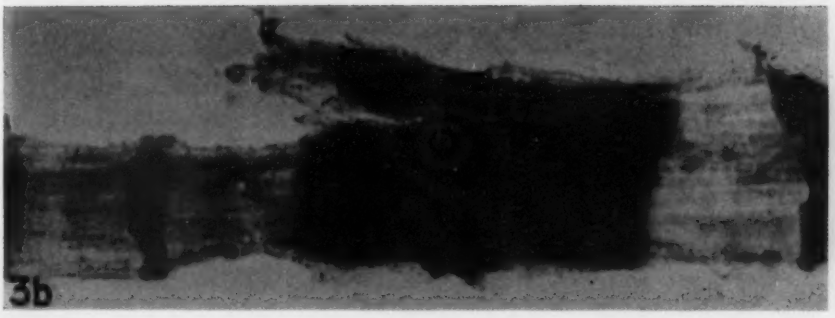
The higher density of the *A* band in electron micrographs of unstained fibrils is in accord with the fact that the refractive index of *A* is higher than that of *I*. Since the myosin filaments pass continuously through both bands, it may be concluded that some other substances occur in much higher concentration in *A* than in *I*. This conclusion is in agreement with the observations of Scott (1932), who found a higher concentration of salts in the *A* band, and of Macallum (1905), who demonstrated a distribution of potassium within the *A* band which is remarkably like the distribution of unstained density as seen in Figure 1. Thus it seems quite evident that the *A* band contains, besides myosin filaments, some substance characterized by a relatively high concentration of salts. This material will be designated as the "*A* substance". Phosphotungstic acid is apparently absorbed in proportion to the density of the *A* substance. It should be noted that phosphotungstic acid forms an insoluble complex with potassium and has been used in the quantitative determination of potassium by Rieben and Van Slyke (1944). This does not mean that the phosphotungstic acid locates potassium specifically for it may react with numerous substances; nevertheless, the observed absorption of this stain in the *A* band is consistent with the conclusion that the *A* substance does contain a relatively high concentration of potassium. That the *A* substance is closely associated with the myosin filaments is indicated by the lack of any observable quantity of interfilamentary material in frayed *A* bands.

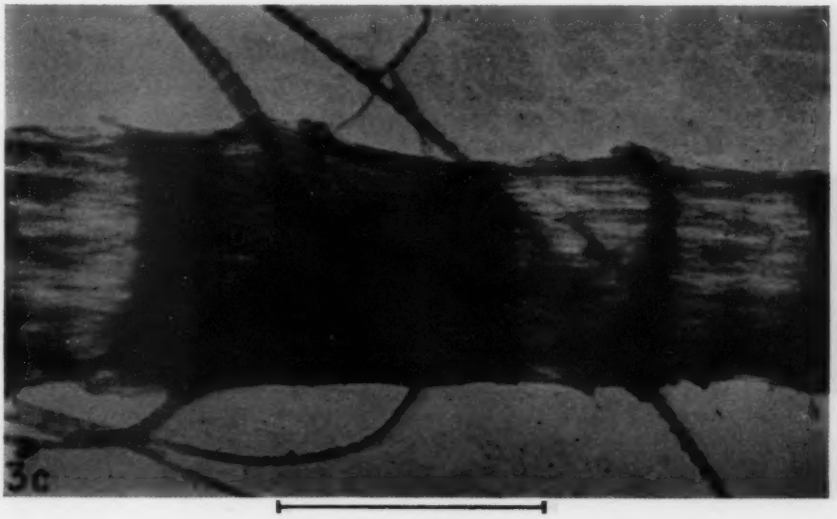
The sharp boundary between the *A* and *I* bands is noteworthy since there is no apparent membrane or other structure to confine the *A* substance. Owing to the limited resolution of the light microscope it had been concluded (Schmidt, 1937) that the transition between the *A* and *I* bands is not abrupt but gradual. Likewise there is no evidence in electron micrographs for any envelope or limiting membrane around the myofibril.

One of the most prominent histological features of striated muscle is the *Z* membrane, the nature of which has been the subject of much discussion in the past. In electron micrographs the *Z* membrane appears to be amorphous material of high staining affinity which cements the myosin filaments together in this region. Frequently in frayed myofibrils the filaments separate laterally but adhere to one another in the region of *Z* and also at *M*. Furthermore, there is a tendency for the filaments to break at *Z* and sometimes at *M*. It has been postulated that the *Z* membrane takes the form of an annular ring about the fibril (see Liang, 1936). Although this could be true, there is no unequivocal electron microscope evidence for such a conclusion. The *Z* membrane appears to consist of interfilamentary material present throughout the fibril and not limited to the periphery. It is definitely not collagenous as was suggested by Häggqvist (1931).

FIGURE 3. Myofibrils from frog sartorius, stretched about 30 per cent, stained with phosphotungstic acid; (*c*) shows collagen fibrils. × 40,000.

PLATE II



*Collagen in muscle*

In the preparation of specimens no effort was made to separate the collagen, which occurs in skeletal muscle in appreciable quantity. However, there is no difficulty in identifying collagen since it has a regular spacing of about 640 Å and displays a characteristic fine structure after staining with phosphotungstic acid (Schmitt, Hall, and Jakus, 1945). Several stained collagen fibrils are visible in Figure 3c. It has been observed that collagen fibrils from rabbit and frog muscle are quite uniform in width (about 500 Å), but the relation of this protein to the muscle structure is not evident in present electron micrographs because of the extensive fragmentation of the muscle.

*Structural alterations in extension and contraction*

Although evidence is not yet sufficient to permit a complete description of the structural alterations associated with extension and contraction, some observations have been made and may be described. Extended sarcomeres were obtained by stretching an excised frog sartorious and fixing it at the stretched length. In some instances the muscle was stimulated electrically during fixation to produce isometric contraction. In the absence of the electrical stimulus, the fixative itself provided a weakly stimulating effect. In other experiments, excised muscles were placed in formalin without restraint, thus producing a state of weak isotonic contraction. Strong isotonic contraction was produced by electrical stimulation of excised muscles before and during fixation. Myofibrils from these muscles were prepared for electron microscope observation in the manner described.

The high concentration of *A* substance toward the ends of the *A* band, resulting in the appearance of the *H* disc, occurs consistently in myofibrils from muscles which have been stimulated (electrically or by the fixative) while held in a state of extension (Figs. 1 and 3). Muscles which are free from tension and are stimulated to contract during fixation do not in general show a division of the *A* band. Myofibrils from such muscles are shown in Figure 2.

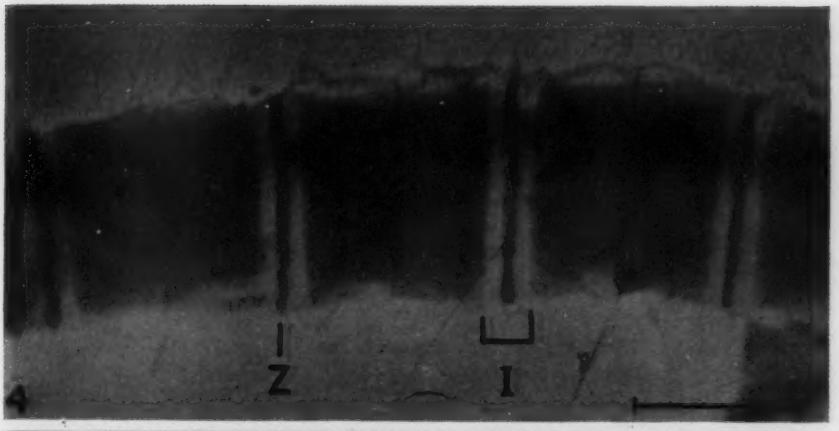
In the myofibrils of muscles which were stretched up to 130 per cent of rest length and fixed at this length, the *A* bands are about the same length as those in muscles which had contracted slightly as a result of fixation. Thus the increase in sarcomere length in stretched and fixed muscles is due mainly to an increase in the length of the *I* band. This observed relative constancy in the length of the *A* band during extension is in agreement with the findings of Buchtal, Knappeis, and Lindhard (1936) who concluded that the *I* band has a relatively low modulus of elasticity.

Electron micrographs of myofibrils from muscles fixed in a state of strong contraction show sarcomere lengths down to about 1 $\mu$ as compared with about 2 $\mu$ in relaxed or weakly contracted muscles. In general the observed sarcomere structure is of two types. The first type, shown in Figure 4, consists of a very narrow *I* band and a quite solid *A* band in which the median *H* disc is absent or very faint. The sarcomere length in Figure 4 is about 1.5 $\mu$, which is about 25 per cent less than the average length in relaxed muscles. The second type of sarcomere structure

FIGURE 4. Myofibril from frog sartorius contracted by electrical stimulation, stained with phosphotungstic acid. × 25,000.

FIGURE 5. Myofibrils from frog sartorius, strongly contracted by electrical stimulation. (*a*) × 25,000 (*b*) × 40,000.

PLATE III



found in strongly contracted muscles is shown in Figure 5. Sarcomere lengths in this case are about 1.2 $\mu$, which is about 60 per cent of the average relaxed length. The *I* band is no longer visible and *Z* appears somewhat wider and more poorly defined than in less strongly contracted fibrils. The region between *M* and *Z* is of uniform density and presumably contains a uniform concentration of *A* substance. In occasional fibrils the *Z* and *M* are distinguishable only with difficulty. Such fibrils may represent a transition stage between the two types of fibrils described. If this is the case, the second type of fibril might result from the accumulation, around the *Z*, of *A* substance which had migrated away from *M*. This stage apparently corresponds to the striation reversal described by Jordan (1933) and earlier by Rollett (1891), who refers to this densely staining *Z* as the *C*, or contraction band.

One aspect of particular significance in strongly contracted sarcomeres is the relative straightness of the myosin filaments, as seen in Figure 5. Since the contracted sarcomeres are as little as 50 per cent of the relaxed length, this can only mean that the filaments themselves shorten in contraction. Similarly, in extension the filaments must individually lengthen. Changes in sarcomere length are not to be associated with any gross spiralling or folding of filaments in the order of dimensions visible in the electron microscope. The myosin filaments are the smallest visible contractile units.

## Isolated Myosin Filaments

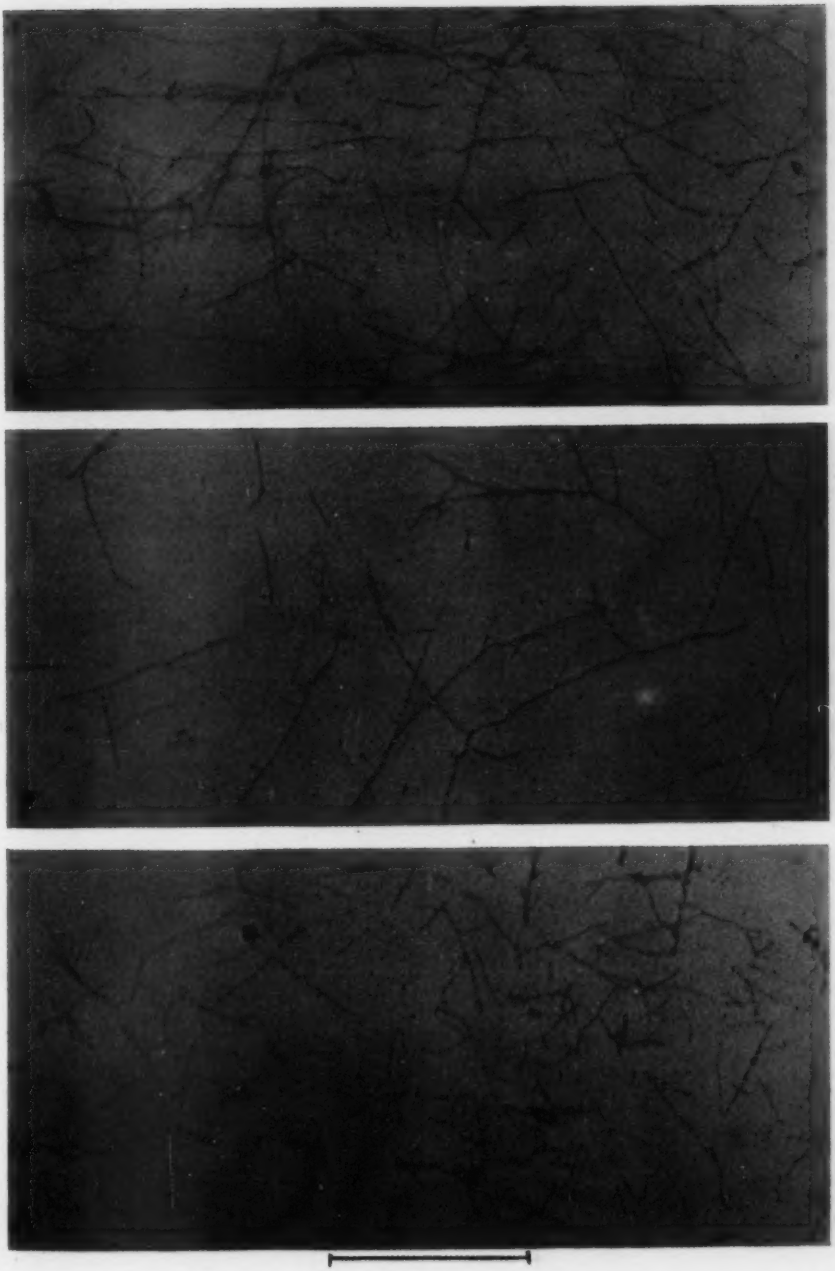
Myosin may be extracted from muscle in weakly alkaline salt solutions and these extracts have been shown to contain filaments visible in the electron microscope (Ardenne and Weber, 1941). In order to determine whether visible differences exist between different myosins, a quantitative study of filaments from various muscles was undertaken.

Extracts of myosin were prepared by a method essentially similar to that described by Greenstein and Edsall (1940). Muscles were removed from the animal immediately after death, trimmed, cut into small pieces and blended in a Waring Blendor with about ten parts of a cold solution of KCl (0.5 M) and $NaHCO_3$ (0.03 M). The suspension was stirred mechanically, at about 4° C., for periods of time varying from seven to twenty-four hours and strained through several layers of closely-woven cheese-cloth. The filtrate, showing strong double refraction of flow, was poured into 8 to 10 volumes of cold distilled water, with constant stirring, and the precipitate which formed was allowed to settle overnight in the cold. Further concentration was accomplished by centrifugation, after removal of the supernatant. The precipitated myosin was washed with cold distilled water, and redissolved by adding powdered KCl crystals to a concentration of about 0.45 M. To further purify the myosin the precipitation and solution was repeated once or twice.

For electron microscope examination, the myosin preparation was diluted to the desired concentration with the KCl–$NaHCO_3$ solution used in the extraction and centrifuged to remove any undissolved protein. A droplet of the solution was

FIGURE 6. Myosin filaments from rabbit leg muscle. × 30,000.
FIGURE 7. Myosin filaments from lobster abdominal muscle. × 30,000.
FIGURE 8. Myosin filaments from clam (Mya arenaria) adductor muscle. × 30,000.

PLATE IV



placed on the supporting film of the specimen grid, the excess removed by blotting, and the film washed with Edsall's solution. The adhering filaments were then fixed and stained, for about one minute, with 0.1 per cent phospho-12-tungstic acid (pH 3 to 5) and washed briefly with water to remove the uncombined phosphotungstic acid. Unstained filaments are poorly defined because of low contrast and, in the absence of the stabilizing effect of the heavy metal ion, appear to be adversely affected by the drying process.

Myosin extracts were made from the muscles of rabbit leg, frog leg, lobster abdomen, scallop adductor (striated part) and clam (Mya) adductor (classified as smooth muscle). Electron micrographs of such myosin preparations show slender filaments similar to those observed in intact myofibrils and resembling those shown by Ardenne and Weber (1941). The filaments have varying lengths and widths and display no tendency to branch or split longitudinally. They are rough in appearance and show fluctuations in density along their length. However, there is no evidence of any regular structural variation which could be interpreted as a significant periodicity. There are no observable changes in density which can be correlated with the *A* and *I* bands, which indicates that the *A* substance has probably been washed out during extraction. This general appearance is very much the same for myosin filaments from the five different animal forms. Typical electron micrographs of myosin filaments from rabbit, lobster, and clam are shown in Figures 6, 7, and 8 respectively.

A statistical study of lengths and widths of myosin filaments from the five selected forms was made. Measurements were taken directly from enlarged prints and in any given print all filaments were measured which could be discerned as complete and individual. Widths can be measured only approximately because of the smallness of this dimension and also because of the roughness of contour. The uncertainty of measurement, however, is significantly smaller than the spread in widths. Results are shown in Figure 9. Each interval in the plot of widths contains the same number of measurable increments and each set of measurements represents about 300 filaments.

TABLE I

*Dimensions of myosin filaments*

| Muscle source | Average width | Average length |
|---|---|---|
| Rabbit leg | 120 Å | 4100 Å |
| Frog leg | 140 Å | 4100 Å |
| Lobster abdomen | 140 Å | 6800 Å |
| Scallop adductor, striated | 130 Å | 5000 Å |
| Clam (Mya) adductor, smooth | 150 Å | 3100 Å |

Nearly all measured widths fall into a narrow range between 50 and 300 Å. There may be some filaments having widths below 50 Å which were not observed because of the resolution and contrast limitations of the electron microscope. However, the quality of the background in the electron micrographs and the fact that all distributions fall off toward small widths indicate that no great quantity of the myosin occurs in this range in electron microscope specimens as prepared.

Between one animal form and another there is very little variation in magnitudes or distribution of widths. There is a slight difference in the averages as shown in Table I, but it is so small as to be scarcely significant.

Filament widths were also measured from electron micrographs of intact myofibrils from frog muscles. The distribution plot resembles very closely the corre-

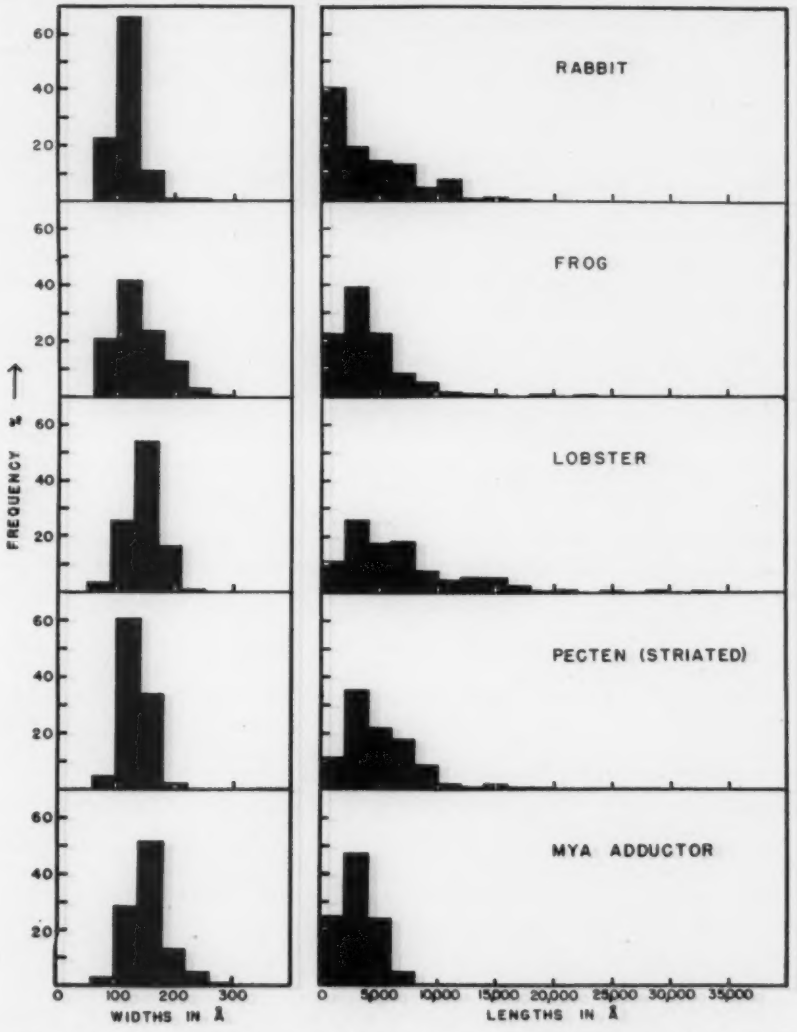



FIGURE 9. Distribution of lengths and widths of myosin filaments from muscles of various animal forms.

sponding plot for frog shown in Figure 9, thus indicating that there is no great difference in the width of filaments in electron micrographs of intact fibrils and myosin extracts.

In comparison to the fair constancy of widths there is a wide distribution of lengths. Most of the measured lengths are below 15,000 Å. Filaments from lobster muscle are in general significantly longer than the others, while filaments from Mya aductor, which is the only smooth muscle studied, are significantly shorter. As a result of the wide range of lengths there is only rough significance in the number-averages given in Table I. The distribution plots are reasonably reproducible under similar conditions.

It is evident from the statistical results that myosin extracts of this type do not constitute a monodisperse system and there is no justification for referring to the filaments as molecules. Widths of filaments apparently do not alter appreciably during extraction. The lengths, however, bear no relation to any observable dimensions in the intact myofibrils. Since most of the lengths represented in the plots of Figure 9 are considerably shorter than are the corresponding sarcomeres, it is apparent that the myosin filaments have been broken at random into shorter segments during the extraction procedure.

Since the myosin filaments seen with the electron microscope are to be identified with the asymmetrical particles responsible for the streaming double refraction of myosin solutions, it is pertinent to compare the results of the methods where possible. Mehl (1938) reported the length of rabbit myosin as 8,500 Å while Edsall (1942) gives a figure of 12,600Å. The second figure is near the upper limit of the distribution plot for rabbit in Figure 9 while the first falls within the range of lengths representing the bulk of the protein. Although no figures have been reported on the other muscles used here, Edsall and Mehl (1940) have described lobster myosin as being more viscous than rabbit myosin at equal concentration and strongly birefringent, which is in qualitative agreement with the electron microscope observation that lobster preparations contain significantly longer fibrils than do those from rabbit.

Ardenne and Weber (1941) do not give the source of the myosin extract from which they made electron micrographs. They state that the filament widths are from 50 to 100 Å although no measurements are tabulated and the micrographs used as illustration contain filaments at least 200 Å in width. The myosin filaments within the limited field of the micrographs which these authors have shown are apparently similar to those described here.

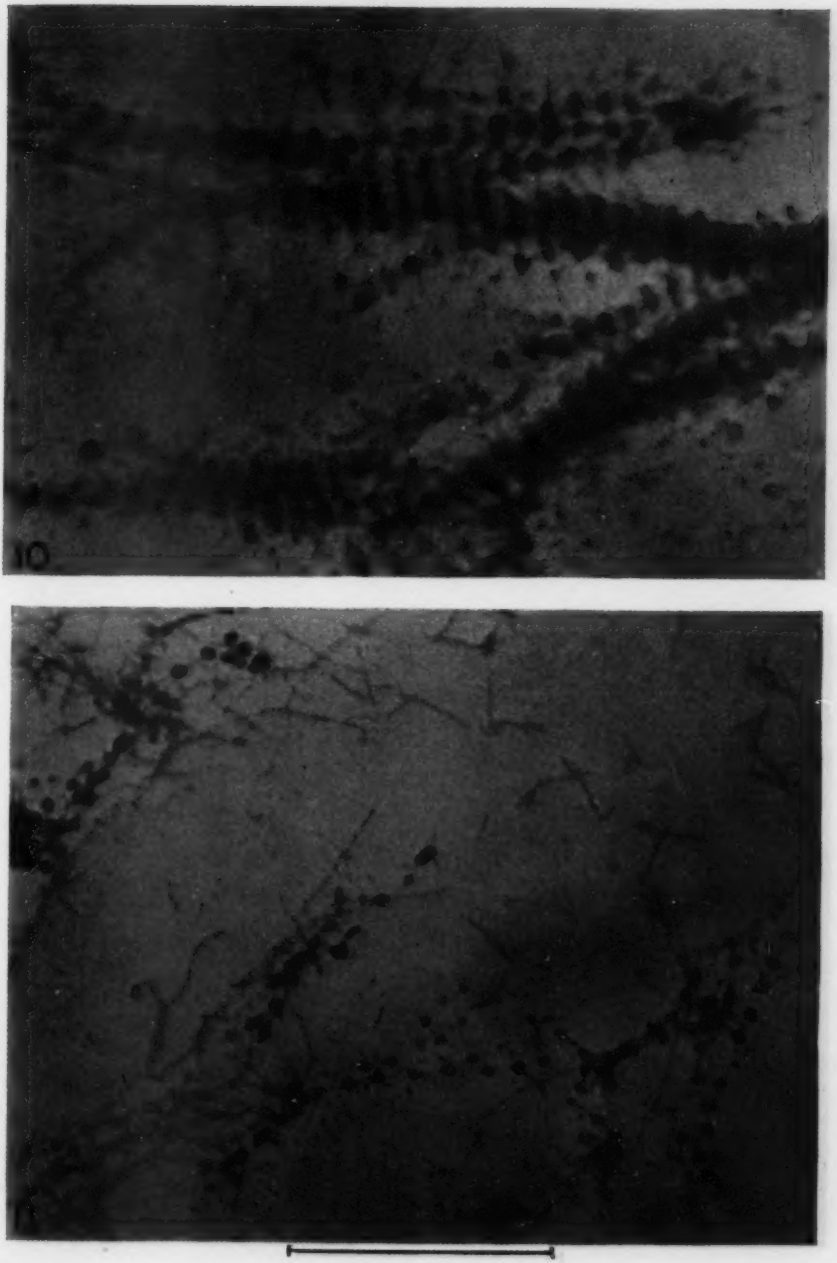
*Paramyosin*

In a recent publication Hall, Jakus, and Schmitt (1945) described the structure of a fibrous muscle globulin which is present in appreciable amount in molluscan smooth muscles, and correlated the observed structure with the x-ray patterns obtained by Bear (1944) from the same material. Since this protein can be identified by electron microscope observation and x-ray diffraction it merits a distinguishing name and is therefore designated as *paramyosin*. Although paramyosin

FIGURE 10. Regular structures from clam adductor muscle after dispersion in Edsall's solution. × 40,000.

FIGURE 11. Fragments from dispersed clam muscle. Darkly staining particles are adhering to fine filaments, presumably myosin. × 40,000.

PLATE V



resembles myosin in some respects, there are definite differences between the two proteins. Paramyosin contains a characteristic axis spacing of about 145 Å. Paramyosin has not been detected in striated muscle while myosin is apparently common to all muscles. Furthermore, the typical needle-shaped fibrils of paramyosin disintegrate in Edsall's solution (0.5 M KCl, 0.03 M $NaHCO_3$) while the myosin filaments remain essentially the same as in fixed intact muscle except as they may be broken into shorter lengths during extraction.

*Clam adductor muscles*

The adductor muscle of the clam differs from the other muscles investigated in that it is classified histologically as "smooth" muscle and contains a large quantity of the fibrous protein, paramyosin. Electron microscope observation has failed to reveal any large periodicity resembling the characteristically banded sarcomere of striated muscle. However, if clam muscle is dispersed in Edsall's solution and centrifuged, there are thrown down fibrous aggregates to which large particles adhere. A typical micrograph of this type of material is shown in Figure 10. The structure consists essentially of bundles of fine filaments (presumably myosin) and darkly staining nodules producing a cross striation with a period of about 1,100 Å. In Figure 11 the bundles have been dispersed, revealing the individual dark nodules which adhere to the fine filaments at more or less regular intervals. The attachment of the dense component at regular intervals along myosin bundles (Fig. 10) is suggestive of a rudimentary structure analogous to the *Z* membrane and sarcomere of striated muscle. As yet, the significance of this structure is not known.

## Discussion

With respect to the existence and disposition of the cross striations, the electron microscope observations on fixed muscle, stained and unstained, are in close agreement with the results obtained with the light microscope (see Jordan, 1933). Confirmatory evidence is presented concerning the *N* bands, *H* discs and the nature of "striation reversal" in strong contraction. Additional information has been added by virtue of the high resolution of the electron microscope. Thus it is possible to say definitely that the *Z* band is not collagenous as suggested by Häggqvist (1931) and to observe directly the myosin filaments and their relation to previously determined structures.

Of fundamental importance is the observation that the myosin filaments extend continuously through the fibril in relatively parallel straight lines. There is no marked disorientation of the filaments in either *A* or *I* bands of fibrils in any state of contraction. Although the optical anisotropy of the *A* band led to the postulate that it contains asymmetric myosin particles, there has always been some doubt as to whether the relatively isotropic *I* band consists of myosin in unoriented state or of some other protein (Weber, 1934). It is now clear that the *I* band consists mainly of well oriented myosin filaments and it is not possible to account for the low birefringence on the basis of gross disorientation. Possibly the difference in anisotropy between the *A* and *I* bands is to be attributed to differences in orientation within the filaments. The only obvious alternative explanation is that the low birefringence in the *I* band is due to partial compensation of the birefringence of the myosin filaments by other components.

No evidence has been found in this investigation for the existence, either in myofibrils or in extracted myosin, of "rodlets" of the specific dimensions postulated by Weber (1934) from polarized light and diffusion experiments. Theoretical difficulties underlying Weber's calculations have been pointed out by Frey-Wyssling (1940) and Schmitt (1944).

Electron micrographs of isolated myosin filaments show that in width and general appearance the filaments are similar to those seen in fixed intact myofibrils. Although myosin filaments from whatever source are essentially the same, the statistical study indicates that they occur in various lengths and are not to be designated as discrete "myosin molecules". However, the length of rabbit myosin reported by Mehl (1938) from streaming double refraction studies is reasonably close to the weight-average length calculated from the distribution curve in Figure 9. No reliable measurement of width is available for comparison with the electron microscope results.

Since myosin filaments in intact fibrils are continuous, the wide range of lengths found in myosin suspensions is noteworthy. It appears that the filaments, while little changed in width, are broken more or less at random during the extraction procedure. The longest filaments approach the sarcomere length and there may be significance in the fact that lobster muscle yields longer filaments than does frog muscle and also has the longer sarcomere. The absence of filaments longer than their corresponding sarcomere is consistent with the observed tendency of the filaments to break at *Z*.

Ziff and Moore (1944), following an extraction procedure similar to that used here, state that their myosin solutions contain a homogeneous substance which forms sharp boundaries in electrophoresis and sedimentation. If homogeneity is meant to denote constancy in particle length, this conclusion is in disagreement with the electron microscope results. In considering the apparent inconsistency it should be noted that, in the electrophoresis and sedimentation of rod-shaped particles of nearly constant diameter, a sharp boundary does not necessarily indicate a constant length. Schramm and Weber (1942) reported that a small fraction of the myosin in extracts has a much higher sedimentation constant than that of the predominant component. No evidence for such a distinct heavy component has been found in the present study unless it represents aggregates of myosin filaments.

The "molecular weight" of myosin has been estimated by Weber and Stöver (1933) to be $0.6 - 1.2 \times 10^6$ and by Ziff and Moore (1944) to be $3.9 \times 10^6$. If a particle weight is calculated from the average dimensions of the filaments in rabbit myosin from Table I, using a density of 1.3 and assuming a circular cross section, the result is $36 \times 10^6$. The results differ by at least a whole order of magnitude. In view of the fair agreement as to filament lengths from streaming birefringence and electron microscope observations, the discrepancy in particle weight is difficult to understand. It may be that the cross section of filaments dried on the supporting film is not circular as assumed in the electron microscope calculation, but it is doubtful whether this assumption could introduce an error large enough to account for the discrepancy. In any event, the significance of all such calculations is questionable inasmuch as the extracts contain particles of widely differing dimensions, in no sense to be considered as molecular entities.

Bear has reported small-angle x-ray diffractions from various muscles. One set of diffractions (Bear, 1944) has been correlated with the structure of para-

myosin fibrils, as determined from electron micrographs (Hall, Jakus, and Schmitt, 1945). This set of diffractions has not been obtained from any striated muscle.

In addition Bear (1945) has reported a second set of small-angle diffractions obtained from a variety of muscles, both smooth and striated. The wide occurrence of this pattern is strongly suggestive that the diffractions originate in the myosin component but it has not been possible to identify in the electron microscope the structure responsible for the diffractions. Furthermore, since the meridional diffractions are orders of 27 Å, it is doubtful whether the structure can be observed directly. Another feature of the diffractions is the occurrence of a periodicity estimated by Bear to be between 350 and 420 Å. Although this dimension is quite large enough for electron microscope resolution, it appears that the pattern is somewhat like the paramyosin pattern in that the large periodicity cannot be discerned unless the axis spacing (27 Å) is well resolved. The fine banded appearance frequently observed in myofibrils is of about the same order of magnitude as the large periodicity, but this may be fortuitous. The x-ray data also indicate a lateral periodicity of about 115 Å which should be large enough for electron miscroscope observation. Although the *average* width of myosin filaments is quite close to this figure, the significance, if any, of this coincidence remains to be determined.

Since the myosin filaments are observed to pursue a straight course through the *A* and *I* bands in fibrils from contracted as well as relaxed muscles, it may be concluded that contractility is a property of the individual filaments in their normal environment in muscle. It seems probable that alterations in length and tension depend on changes within the filaments in response to changes in the chemical environment. In seeking a description of the contractile mechanism, due consideration must be given to the role played by the *A* substance and the adenosine triphosphate. The electron microscope technique provides a promising method for studying these structures and processes. It may be expected that further correlation of the x-ray, electron microscope, polarization optical and chemical evidence will contribute greatly to an understanding of the nature of contractility.

## Summary

1. Electron micrographs were made of myofibrils isolated from frog and other skeletal muscles fixed in formalin. The structure with respect to the location and disposition of the principal cross striations is in good agreement with that previously determined from histological studies.

2. The myofibrils are composed of bundles of myosin filaments ranging in width from about 50 to 250 Å and extending continuously and in relatively straight lines through the isotropic and anisotropic bands in both the extended and contracted states. The anisotropic bands also contain material of high electron scattering power and affinity for phosphotungstic acid. The distribution of this "*A* substance" changes with contraction in characteristic fashion. The evidence indicates that the myosin filaments are the contractile units.

3. While the myosin filaments have an indefinite length in the intact fibril, they are fragmented extensively during extraction in weakly alkaline salt solutions (method of Greenstein and Edsall). Filaments from such extracts have fairly uniform widths (50 to 250 Å) but highly variable lengths, in general below 15,000 Å. Filaments from rabbit, frog, lobster, scallop and clam muscles are similar in appear-

ance; widths are fairly uniform but lengths vary significantly from one form to another.

4. The relation of these findings to physical chemical data previously obtained by others on similar myosin extracts is discussed.

5. In the one smooth muscle examined (clam adductor) no striations comparable to those of skeletal muscle fibrils were found. However, a regular structure with a period of about 1,100 Å was observed.

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# EFFECT OF WATER CURRENTS UPON THE ATTACHMENT AND GROWTH OF BARNACLES [1]

F. G. WALTON SMITH

*University of Miami Marine Laboratory* [2]

## Introduction

There exists a considerable body of published work dealing with peculiarities in the distribution of sessile marine invertebrates in general, and, although experimental evidence is incomplete some speculation has been offered as to the manner in which their distribution is limited by various factors of the environment which influence their attachment and growth. The object of the present studies was to investigate the action of the water current factor by experimental methods, with particular reference to the species of barnacles which are most abundant in the Miami area.

Much of the earlier work on sessile marine animals has been reviewed by Mc Dougall (1943), and need not be considered here in detail. A bibliography of investigations dealing with sessile organisms from the point of view of ship fouling is given by Neu (1933), and an excellent general account of marine fouling by Visscher (1928) who does not, however, include more than a brief reference to the effect of water currents.

The effects of environmental factors have been deduced from the results of ecological surveys by a number of investigators. Stephenson and his co-workers, in a series of publications entitled *The South African Zone and Its Relation to Water Currents* (see bibliography), Fischer-Piette (1928, 1928a), Pierron and Huang (1926), and Prennant and Teissier (1924) have described the populations of rocky shores and the distribution of sessile organisms in general, but with scanty reference only to the relation between barnacle attachment and the velocity of water currents. Stephenson and Bright (1938) and Stephenson and du Toit (1937), however, note the absence of certain species of barnacles from surfaces exposed to heavy wave action.

The possibility of water movement influencing barnacle attachment is suggested by Fischer-Piette (1932) in his reports on surveys of the shores of the English Channel. Shelford and Towler (1925), Towler (1930), and Newcombe (1935) have paid particular attention to barnacles in associations of Balanus-Littorina and Balanus-Mytilus and draw attention to the more luxuriant growth of barnacles exposed to strong water currents, particularly in shallow water. The role of water currents is also mentioned briefly in accounts of ecological surveys by Rice (1930), Moore (1935a), and Moore and Kitching (1939), who stress the part played by

[1] These experiments were conducted while the author was engaged by the Woods Hole Oceanographic Institution in investigations under contract with the Bureau of Ships, Navy Department, which has given permission to publish the results. The opinions contained herein are those of the author and do not necessarily reflect the official opinion of the Navy Department or of the Naval service at large.

[2] Contribution number 301 from the Woods Hole Oceanographic Institution.

them in the provision of an adequate food supply, as well as the adverse effects of wave action.

Experimental investigation has yielded more direct information. Moore (1933) studied the effect of currents upon the attachment of cyprid larvae with particular regard to orientation, but gives no estimate of current velocities which limit attachment. The related phenomena of wave action are considered by Moore in a separate paper (1939).

Evidence as to the effect of water currents is also afforded by the study of ships and other objects moving through the water. The economic importance of ship-bottom fouling has stimulated this kind of observation. Heutschel (1934) and Neu (1932) state that vessels collect barnacles most readily when in harbor and when subjected to relatively less water movement. Visscher (1930) further observes that barnacles are usually killed during ocean passages of about 500 miles. Hiraga (1934) showed experimentally that, in the laboratory, 10 to 17 day old barnacles were lost during a five day exposure of test planks to water currents, whereas twenty-four day old barnacles remained alive and intact. In a later paper of Visscher (1938) he concludes that barnacles are not distributed by ships. This is contrary to the views of Fischer-Piette (1929, 1935) who expresses the opinion that *Balanus amphitrite* found by him on the coast of France, was brought from the tropics on the hulls on ships. Later observations by Phelps (1942) on test panels support the conclusion that excessive water currents inhibit the attachment and growth of barnacles.

The extensive observations of McDougall on the pile fauna at Beaufort include the results of exposing various types of experimental collecting apparatus. His experiments on the effect of water currents indicate that fewer barnacles settle and that growth is slower in more rapid currents. His results did not, however, give an accurate indication of limiting or optimum velocities to which his collectors were exposed.

The experiments which form the subject of the present account are divided into a study of the effects of water currents upon the attachment of cyprid larvae, and into a consideration of the effects of water currents upon the organisms following attachment. Under natural conditions of fluctuating tidal currents and wind drifts it is conceivable that a cyprid might attach during a period of minimum water flow in a particular region and that, once attached, the maximum flow would be insufficient to detach it or to inhibit its growth. The aim of the investigation was to establish the limiting velocities of current for initial and continued attachment and growth.

The advice and suggestions of Dr. Alfred C. Redfield of the Woods Hole Oceanographic Institution are gratefully acknowledged. The writer is also indebted to Mr. Frank L. LaQue of the Development and Research Division, International Nickel Company, Inc. and to Dr. William F. Clapp for permission to include in this paper data from their observations at Kure Beach, N. C. Acknowledgments are also due Mr. D. S. Reynolds, Mr. James Gregg, Mr. Alexander Frue, and Mr. Charles Weiss for their assistance at various times.

## Methods

In order to study the effects of water currents two types of apparatus were designed which would permit the movement of sea-water relative to the experimental

surface at predetermined and variable velocities. The first consisted of a rotating disc, immersed in the sea. The second type of apparatus consisted of a series of glass tubes of varying cross-sectional diameter. The rotating disc was employed both in the study of initial attachment of barnacles and in the study of growth subsequent to attachment. The glass tubes of graded diameter were employed in the observations on initial attachment only, as a check against results obtained with the rotating disc.

## Water Currents and Attachment on the Rotating Disc

The rotating disc apparatus as used in the experiments here described is essentially similar to a machine described by LaQue (1943) for the purpose of studying the effect of sea-water currents upon corrosion of metal samples, with certain changes in design appropriate to the particular use to which it was put. The modified machine consists of a vertical shaft rotated by means of an electric motor and a system of belts and pulleys. The disc is attached to the shaft by means of a flange and small brass screws and is maintained in a position several inches below low water spring tide. At velocities of rotation used in the experiment no cavitation occurred at this depth.

By changing the arrangement of pulleys the speed of rotation may be varied up to approximately 1750 r.p.m. The rate of movement in knots of any portion of the disc relative to an imaginary stationary body of water is readily calculated as approximately $R \times D/370$, where R is the number of revolutions per minute and D the diameter in inches. This velocity is nominal, however, and does not accurately represent the rate of relative movement between the disc and the water close to its surface. Frictional drag and centrifugal forces produce vortex movements which cause water to flow in a close spiral roughly parallel to the plane of the disc. As a result, the actual flow of water at any part of the disc's surface is less than the nominal rate calculated from the velocity of rotation. Size of the discs used varied up to 28 inches in diameter.

Experimental procedure consisted of bolting a new rotating disc on the shaft and setting the machinery in continuous operation. The disc was removed and examined at suitable intervals. At the same time a similar stationary disc was immersed at a point nearby the rotating disc, at the same depth and resting similarly in a horizontal plane. Observations on the second disc acted as a control for such factors as abundance of organisms, nature of the surface of the disc, etc. The apparatus was used at the edge of a covered slip at the Miami Beach Boat Corporation, where fouling is usually severe. The predominant organism at most times of the year is *Balanus amphitrite niveus* Darwin and this was accordingly selected for observation.

### *Results*

The results of the first rotating disc experiment indicated that the approximate minimum current velocity required to prevent barnacle attachment is 1.1 knots. The disc used was 13 inches in diameter and was rotated at 540 r.p.m. The disc, together with its stationary control, are shown in Figure 1 as they appeared at the end of this experiment. Five barnacles attached to the center of the rotated disc during 23 days but did not grow beyond 2 mm. in greatest width. The limiting velocity was calculated from the ¾ inch diameter of the circle to which the barnacle attachments were restricted (Table I).

TABLE I

*Effect of water currents upon attachment of Balanus amphitrite as observed on the lower surface of a horizontal rotating disc*

| Disc | Date of start | Duration of rotation (Days) | Speed of rotation (r.p.m.) | Maximum diameter at which attachment occurred (Inches) | Maximum nominal velocity at which attachment occurred (Knots) |
|---|---|---|---|---|---|
| 1 B | 8/20/43 | 23 | 540 | ¾ | 1.1 |
| 4 A | 11/4/43 | 16 | 192 | 2 | 1.0 |
| 6 A | 12/7/43 | 14 | 134 | 2½ | 0.9 |
| 7 A | 1/5/44 | 19 | 60 | 8 | 1.3 |
| Average | — | — | — | — | 1.1 |

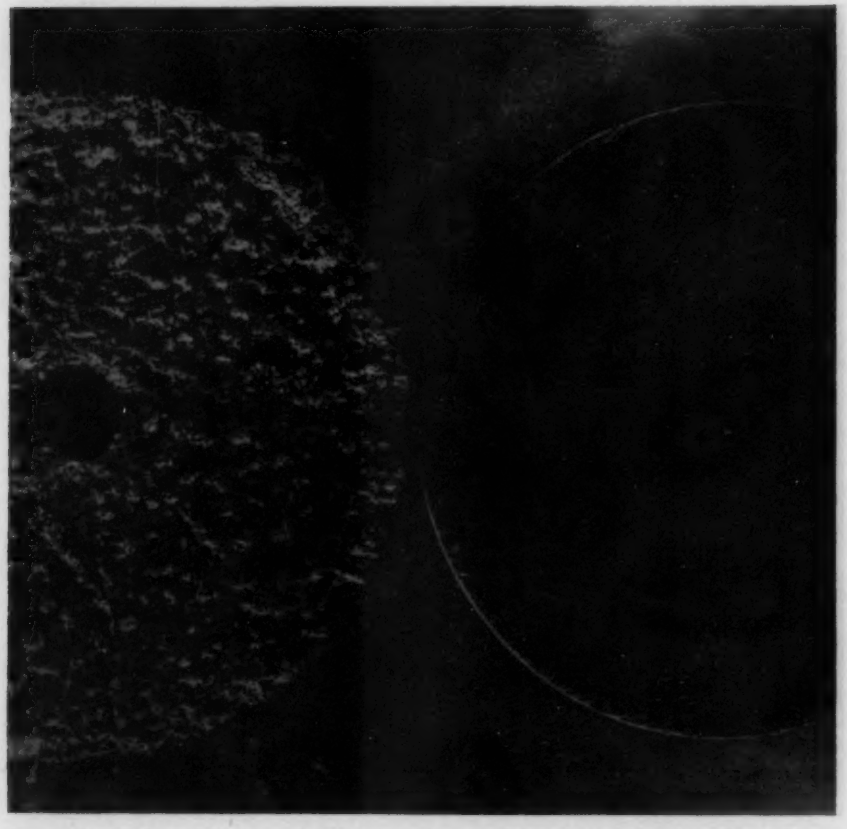

FIGURE 1. Surface of disc rotated at 540 r.p.m. for a period of 23 days, together with stationary control. Diameter of disc, 13 inches.

In order to establish the limiting velocity with greater accuracy the experiment was repeated on three other occasions at slower speeds of rotation, with the results shown in Table I and Figure 2. The nominal velocity limiting barnacle attachment as shown by these experiments varied from 0.9 knots to 1.3 knots with an average of 1.1 knots. In addition to the adverse effect upon barnacle attachment it was noticed that slime film development was greatly reduced on the rotated disc.

## Attachment in Glass Tubes

Owing to the difficulty of accurately measuring the rate of water flow along the surface of the rotating disc, an independent method of investigating the relation between water currents and fouling incidence was used. In this case sea water was passed through sections of glass tubing of varying diameter (Fig. 3). Comparison of fouling incidence in each sector was made with the varying linear velocity of the water, calculated on the assumption that linear rate of flow through tubes varies inversely as the square of the diameter of cross-section. In order to estimate errors which might be introduced by virtue of turbulent flow, carmine was introduced into the water during a preliminary test run. It was observed that flow in the main portion of each section was smooth. Areas near to the joints, where turbulence was noted, were not included in the experimental observations of fouling incidence.

The tubes were approximately eight inches long and of 5 cm., 3.7 cm., 2.8 cm., 2.2. cm., and 1.4 cm. internal diameter respectively and were joined by means of fitted rubber stoppers. Attachment of barnacle larvae was encouraged by excluding direct sunlight. The water flow was provided by means of a centrifugal pump, with the experimental tubes arranged on the inlet side in order to avoid possible mechanical damage to the larvae.

While the apparatus was in operation the rate of flow was checked daily by volumetric measurement at the outlet. At the conclusion of each test run the tubes were carefully examined for signs of fouling.

### *Results*

Observations on the glass tubes showed that limiting velocities for attachment of *Balanus amphitrite* were between 0.5 and 1.0 knot. During the first experiment with this apparatus, sea water was run through it at a rate between 13 and 16 liters per minute, on a catwalk extending over the water at the University of Miami Marine Laboratory. Fluctuations in the height of tide and consequently the suction head of the seawater pump gave rise to this fluctuation in current velocity. Barnacles attached in the two larger tubes within ten days after beginning the experiment. From the calculated linear velocity of the narrower of these tubes it appeared that barnacles are not prevented from attachment by currents varying between 0.5 and 0.6 knot (Table II). In the next smaller tube where no barnacles attached the rate of flow varied from 0.8 to 1.0 knot. The limiting velocity therefore lies between 0.5 knot and 1.0 knot. The appearance of the tubes at the end of a similar experiment is illustrated in Figures 3 and 4. The upper figure of the limits is lower than the critical velocity as measured on the rotating disc. However, in view of the difference in hydrodynamical conditions involved in the two methods, which was discussed previously, the results appear to be reasonably consistent.

FIGURE 2. Portion of disc rotated at 192 r.p.m. for a period of 16 days, showing barnacles attached at center. Diameter of disc, 24 inches.

FIGURE 3. Series of five tubes of increasing cross-sectional diameter. Shows development of barnacles in wider tubes only, following prolonged passage of sea-water.

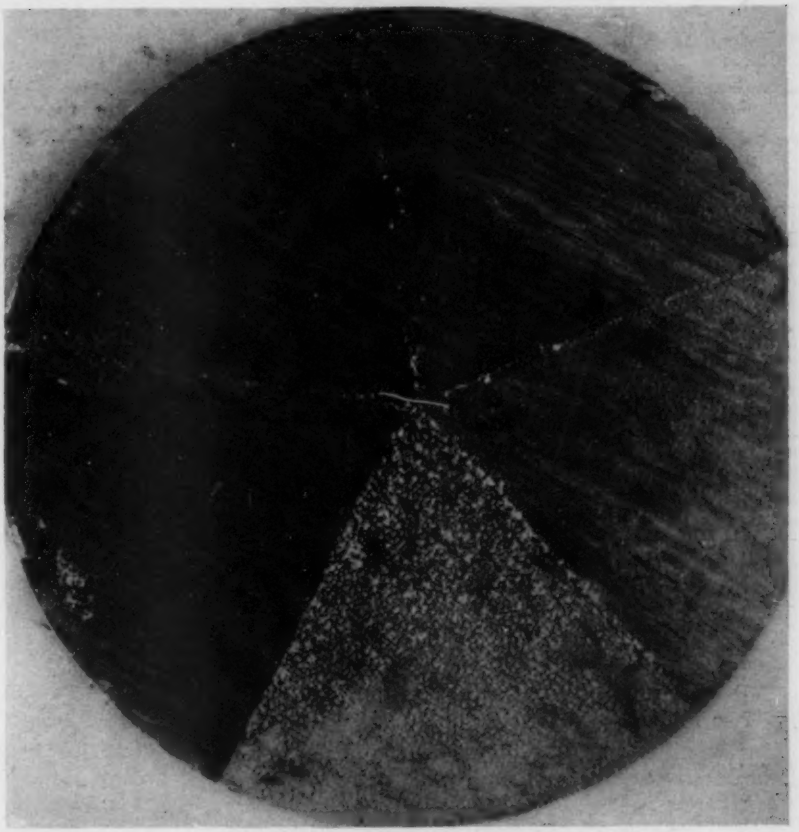

FIGURE 4. Surface of disc following 16 days of stationary immersion beginning January 8, 1944. Individual sectors of the disc were exposed for periods of 6 hrs., 1 day, 2 days, 5 days and 16 days. Diameter of disc, 28 inches.

At a later date the glass tubes were exposed at Kure Beach, N. C., by Mr. F. L. LaQue and Dr. W. Clapp, under conditions allowing a more constant flow of sea water. By reducing the flow of water in successive experiments the critical velocity for attachment was confined more closely between 1.1 and 1.3 knots for *Balanus improvisus* and below 0.8 knot for *B. eburneus*. These results are included in Table II with the permission of LaQue and Clapp.

Confirmatory results have been reported by Turner (1945) from an experiment recently conducted at Kure Beach in which a system of steel pipes of graded diameter was exposed to sea water circulation for 98 days. Fouling with mussels, barnacles, and tube worms occurred where the current velocity was one knot or less but not where it was 1.8 knots or greater.

TABLE II

*Attachment of Balanus species upon vertical glass tubes of graded diameter during the passage of sea-water*

| Period and place | Rate of flow. Liters per minute | Equivalent velocity in glass tubes (Knots) | | | | |
|---|---|---|---|---|---|---|
| | | 1.4 cm. diameter | 2.2 cm. diameter | 2.8 cm. diameter | 3.7 cm. diameter | 5.0 cm. diameter |
| 11/17/43 to 11/27/43 Miami Beach | 13–16 | 3.0–3.7 — | 1.2–1.6 — | 0.8–1.0 — | 0.5–0.6 *B. amphitrite* | 0.2–0.3 *B. amphitrite* |
| 5/20/44 to 7/12/44 Kure Beach* | 21 | 5.3 — | 2.3 — | 1.4 — | 0.8 Balanus sp. | 0.4 Balanus sp. |
| 7/12/44 to 9/18/44 Kure Beach* | 12.7 | 3.2 — | 1.3 — | 0.8 Balanus sp. | 0.5 Balanus sp. | 0.2 Balanus sp. |
| 9/18/44 to 11/25/44 Kure Beach* | 10.8 | 2.7 — | 1.1 *B. improvisus* | 0.7 *B. improvisus* | 0.4 *B. eburneus* *B. improvisus* | 0.2 *B. eburneus* *B. improvisus* |

* Observations by W. F. Clapp and F. L. LaQue.

## GROWTH ON THE ROTATING DISC

For the purpose of studying the effect of water currents upon barnacles already attached the rotating disc procedure was modified. Both rotating and stationary discs were allowed a preliminary period of stationary immersion during which barnacles became attached. Equal sectors of the disc were temporarily protected by means of cloth attached with thumb tacks. The cloth was removed from each sector after increasing intervals of time with the result that at the end of the preliminary period of immersion the maximum age of the organisms attached to the different parts of the disc varied from 16 days to 6 hours. The barnacles on each sector were then counted and the maximum diameter of the largest barnacle in each sector was measured.

Following the period of stationary immersion the control disc was allowed to remain in a stationary position and the other was placed on the rotating shaft for a further period. Both discs were examined and the number of barnacles per square inch and their greatest diameter were recorded separately for each sector and for areas at successive distances from the center of the disc.

### *Results*

The first experiment of this series provided an initial 16 days' period of stationary immersion during which both experimental and control discs became seeded

with barnacles. At the end of this period barnacles had been attached on the five sectors for maximum periods of 6 hours, 1 day, 2 days, 5 days, and 16 days respectively (Fig. 4). Immediately following the period of stationary growth, the experimental disc was rotated at an angular velocity of 60 r.p.m. for 19 consecutive days, while the control disc was allowed to remain in a stationary position for the same period. The appearance of both discs at the end of the experiment is shown in Figures 5 and 6.

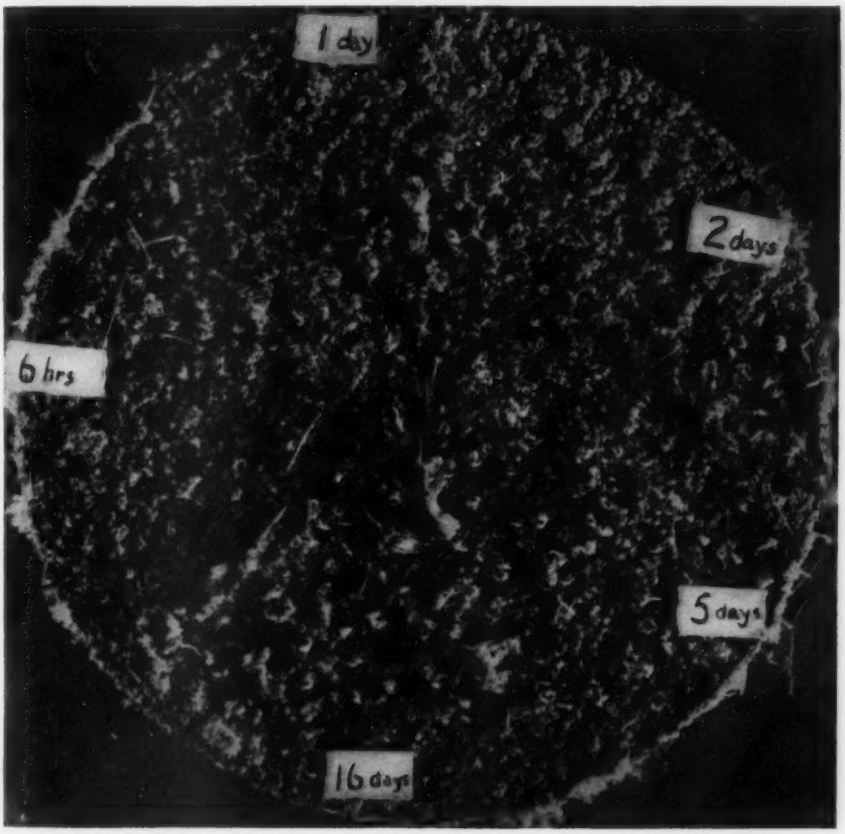


FIGURE 5. Same disc as in Figure 4 after further period of 19 days stationary immersion. Control to disc shown in Figure 6.

Examination of the discs at the conclusion of the test showed that while increasing current velocities diminished the growth rate and finally brought about loss of attachment, the weaker currents appeared to enhance growth. Thus, when rotated on Sector I, where growth had continued for 6 hours before rotation, *B. amphitrite*

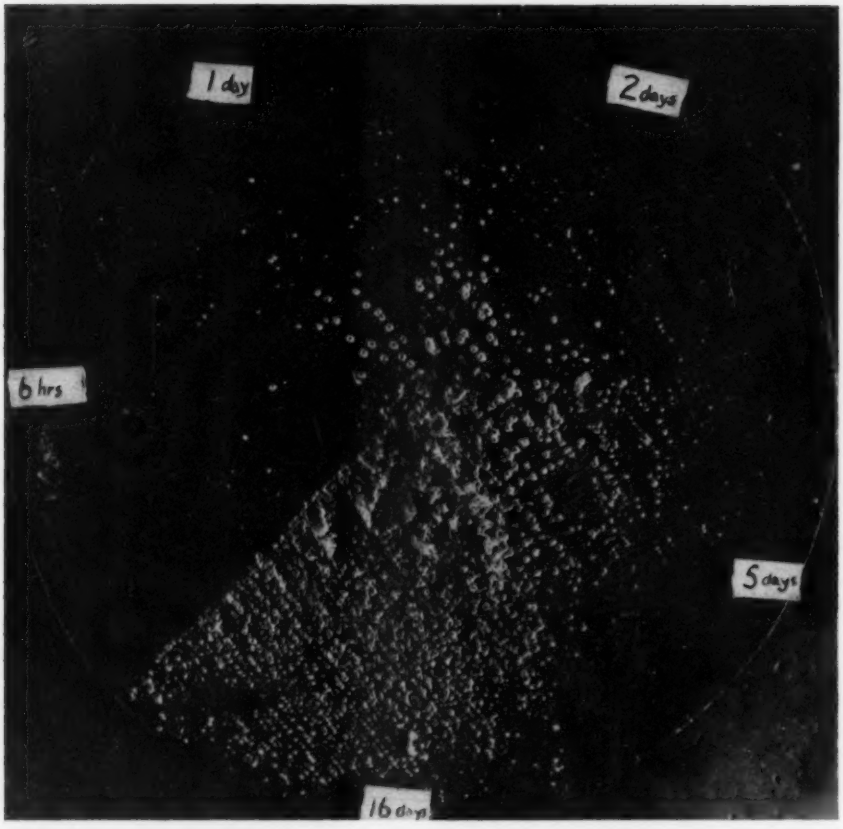


FIGURE 6. Similar disc to that of Figure 4 after period of 19 days rotation at 60 r.p.m. Age of barnacles before rotation is marked on each sector.

neither continued development nor remained attached at diameters beyond 16 inches, corresponding to a current of 2.7 knots (Table III). Within Sector VI, however, with barnacles originally 16 days old, a small number remained attached even at the outer edge of the disc, where the water flow approximated 4.7 knots. On the intervening sectors with barnacles of intermediate ages, the current velocities bringing about complete loss of attachment showed values between these extremes. Probably because of further attachments on the stationary control, the density of attachments on all sectors of the rotated disc was below that of the control.

The effects upon growth rate are illustrated by the observations in Table IV. On Sector I, within a 4 inch diameter, equivalent to 0.7 knot, barnacles reached a maximum width of 8 mm., compared with growth on the control of only 7 mm. At 2.3 knots, growth was reduced to only 3 mm. and at 2.7 knots, no barnacles re-

TABLE III

*Effect of water currents upon adhesion of barnacles of different ages when subjected to 19 days rotation upon a submerged disc, as shown by density of attachment in number per square inch. Experiment initiated January 8, 1944*

| Position on disc | | Age before rotation | | | | |
|---|---|---|---|---|---|---|
| Diameter (Inches) | Nominal water velocity (Knots) | Sector I (6 hours) | Sector II (1 day) | Sector III (2 days) | Sector IV (5 days) | Sector V (16 days) |
| 0–28* | 0 | Dense | Dense | Dense | Dense | Dense |
| 4 | 0.7 | 1.7 | 1.7 | 1.7 | 2.0 | |
| 6 | 1.0 | 0 | 0.4 | 0.9 | 1.7 | |
| 8 | 1.3 | 0 | 0.4 | 1.0 | 1.3 | |
| 10 | 1.7 | 0.1 | 0.4 | 1.0 | 1.0 | Too crowded to count |
| 12 | 2.0 | 0.2 | 0.4 | 0.6 | 1.9 | |
| 14 | 2.3 | 0.1 | 0.4 | 0.9 | 1.3 | |
| 16 | 2.7 | 0 | 0.4 | 0.6 | 0.9 | |
| 18 | 3.0 | 0 | 0.1 | 0.2 | 0.6 | |
| 20 | 3.3 | 0 | 0 | 0.1 | 0.4 | |
| 22 | 4.0 | 0 | 0 | 0 | 0 | |
| 28 | 4.7 | Few in cracks | 0 | Few in cracks | Few in cracks | Few |

* Stationary control.

mained on the sector. At intermediate current velocities conditions of growth lay between these two extremes, with normal growth occurring between 0.7 and 1.7 knots.

Similar observations were made upon barnacles of greater initial age within the remaining sectors. Thus, on Sector IV, where development had continued for a maximum period of 5 days before rotation, barnacles showed enhanced growth rate in currents up to one knot, normal growth similar to that of the control up to 2 knots, and almost complete cessation of growth at 3.3 knots. No barnacles remained on this sector at 4 knots.

Similar conditions of enhanced growth at low velocities, normal growth at intermediate velocities and cessation of growth with loss of attachment at higher velocities were observed on the remaining sectors, with barnacles of different initial ages.

The anomalous absence of enhanced growth on Sector V is probably due to the crowded condition of the barnacles which had become attached during a 16 day stationary period. The one day old barnacles were apparently less affected by the

TABLE IV

*Effect of water currents upon growth of barnacles of different ages when subjected to 19 days rotation upon a submerged disc, as shown by diameter of largest barnacle in millimeters. Experiment initiated January 8, 1944*

| Position on disc | | Age and size before rotation | | | | |
|---|---|---|---|---|---|---|
| Diameter (Inches) | Nominal water velocity (Knots) | Sector I 6 hours (<1 mm.) | Sector II 1 day (<1 mm.) | Sector III 2 days (<1 mm.) | Sector IV 5 days (2 mm.) | Sector V 16 days (5 mm.) |
| 0–28* | 0 | 7 mm. | 7 mm. | 8 mm. | 8 mm. | 9 mm. |
| 4 | 0.7 | 8 mm. | 8 mm. | 8 mm. | 9 mm. | None more than 8 mm. |
| 6 | 1.0 | — | 8 mm. | 9 mm. | 9 mm. | |
| 8 | 1.3 | — | 8 mm. | 8 mm. | 8 mm. | |
| 10 | 1.7 | 6 mm. | 7 mm. | 6 mm. | 8 mm. | |
| 12 | 2.0 | 5 mm. | 5 mm. | 6 mm. | 8 mm. | |
| 14 | 2.3 | 3 mm. | 6 mm. | 4 mm. | 6 mm. | |
| 16 | 2.7 | — | 5 mm. | 3 mm. | 5 mm. | |
| 18 | 3.0 | — | 1.5 mm. | 1.5 mm. | 4 mm. | 7 mm. |
| 20 | 3.3 | — | — | 1.5 mm. | 2.5 mm. | 6 mm. |
| 24 | 4.0 | — | — | — | — | 6 mm. |
| 28 | 4.7 | 1.5 mm. | — | 1.5 mm. | 4 mm. | 5 mm. |

* Stationary control.

inhibitory action of water currents than the two day old barnacles. Otherwise, the adverse effects of water currents increased with their velocity, and decreased with the initial age of the barnacles.

On all sectors a few barnacles continued to grow at the perimeter of the disc where the presence of cracks provided local flow pockets with relatively still water.

The first experiment demonstrated an actual loss of barnacles at current velocities having critical values for the different initial ages of barnacles. In order to determine the time required for the loss of attachment to occur, a second experiment was carried out in which the disc was examined, not only at the beginning and end of the rotation period, but also at intervening times. The density of attachment and maximum size of barnacles were observed at each examination, as in the previous experiment.

The experiment was carried out during a period of heavy barnacle set. At the end of the stationary period, extending from February 19 to March 1, 1945, the density of attachments upon each sector, and particularly upon the sectors longest exposed, was very much greater than in the previous experiment.

TABLE V

*Density of barnacle attachment compared with rate of flow of water relative to the surface of a rotating disc, expressed in number per square inch* *
(*Figures in brackets indicate density upon a stationary panel serving as control;* D, *over 75 per square inch*)

| Stationary growth period | Diameter of disc (Inches) | Velocity (Knots) | Period of rotation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 hrs. | 1 hr. | 12 hrs. | 3 days | 5 days | 7 days | 9 days | 11 days |
| Sector I | Control | (0) | (30) | (30) | (30) | (30) | (30) | (60) | (D) | (D) |
| | 4 | 1.5 | 30 | 25 | 25 | 20 | 15 | 10 | 8 | 5 |
| | 8 | 3 | 30 | 30 | 20 | 20 | 15 | 10 | 8 | 6 |
| 6 hrs. | 12 | 5 | 30 | 30 | 20 | 20 | 15 | 6 | 6 | 6 |
| | 16 | 6.5 | 30 | 30 | 20 | 20 | 15 | 6 | 6 | 5 |
| | 20 | 8 | 30 | 30 | 20 | 20 | 15 | 4 | 4 | 2 |
| | 24 | 10 | 35 | 30 | 20 | 20 | 12 | 4 | 4 | 2 |
| Sector II | Control | (0) | (30) | (30) | (30) | (30) | (30) | (60) | (D) | (D) |
| | 4 | 1.5 | 30 | 30 | 30 | 30 | 20 | 15 | 15 | 8 |
| | 8 | 3 | 30 | 30 | 30 | 30 | 20 | 15 | 15 | 12 |
| 12 hrs. | 12 | 5 | 30 | 30 | 30 | 30 | 20 | 15 | 15 | 6 |
| | 16 | 1.5 | 30 | 30 | 30 | 30 | 20 | 15 | 15 | 6 |
| | 20 | 8 | 30 | 30 | 30 | 30 | 20 | 10 | 10 | 5 |
| | 24 | 10 | 30 | 30 | 30 | 10 | 7 | 7 | 7 | 4 |
| Sector III | Control | (0) | (30) | (30) | (30) | (30) | (30) | (60) | (D) | (D) |
| | 4 | 1.5 | 30 | 30 | 30 | 20 | 20 | 12 | 12 | 10 |
| | 8 | 3 | 30 | 30 | 30 | 20 | 20 | 12 | 12 | 12 |
| 1 day | 12 | 5 | 30 | 30 | 30 | 20 | 12 | 10 | 10 | 10 |
| | 16 | 6.5 | 30 | 30 | 30 | 20 | 12 | 10 | 10 | 6 |
| | 20 | 8 | 30 | 30 | 30 | 20 | 12 | 7 | 7 | 5 |
| | 24 | 10 | 30 | 30 | 30 | 7 | 5 | 5 | 4 | 4 |
| Sector IV | Control | (0) | (30) | (30) | (30) | (40) | (40) | (50) | (D) | (D) |
| | 4 | 1.5 | 30 | 30 | 30 | 20 | 20 | 17 | 17 | 15 |
| | 8 | 3 | 30 | 30 | 30 | 24 | 20 | 20 | 20 | 19 |
| 2 days | 12 | 5 | 30 | 30 | 30 | 24 | 20 | 20 | 15 | 9 |
| | 16 | 6.5 | 30 | 30 | 30 | 24 | 20 | 20 | 15 | 8 |
| | 20 | 8 | 30 | 30 | 30 | 15 | 10 | 10 | 10 | 7 |
| | 24 | 10 | 30 | 30 | 30 | 10 | 7 | 7 | 6 | 6 |
| Sector V | Control | (0) | (30) | (30) | (30) | (50) | (50) | (D) | (D) | (D) |
| | 4 | 1.5 | 35 | 35 | 35 | 32 | 32 | 32 | 30 | 30 |
| | 8 | 3 | 35 | 35 | 30 | 30 | 30 | 30 | 30 | 30 |
| | 12 | 5 | 35 | 35 | 30 | 30 | 30 | 30 | 30 | 24 |
| 5 days | 16 | 6.5 | 35 | 35 | 30 | 30 | 30 | 30 | 30 | 15 |
| | 20 | 8 | 35 | 35 | 30 | 30 | 30 | 30 | 30 | 15 |
| | 24 | 10 | 35 | 35 | 25 | 25 | 25 | 25 | 25 | 15 |
| Sector VI | Control | (0) | (70) | (70) | (70) | (70) | (D) | (D) | (D) | (D) |
| | 4 | 1.5 | 75 | 75 | 75 | 75 | 75 | 75 | 75 | 50 |
| | 8 | 3 | 75 | 75 | 75 | 70 | 70 | 70 | 70 | 30 |
| 10 days | 12 | 5 | 75 | 75 | 75 | 70 | 70 | 70 | 70 | 30 |
| | 16 | 6.5 | 75 | 75 | 75 | 70 | 70 | 70 | 70 | 25 |
| | 20 | 8 | 75 | 75 | 75 | 70 | 70 | 70 | 70 | 20 |
| | 24 | 10 | 75 | 75 | 75 | 70 | 70 | 70 | 70 | 20 |

* Experiment initiated 2/19/45.

Observations showed that, as in the previous experiment, losses in attached barnacles occurred roughly in proportion to the current velocity, although at no point of the disc was the entire set lost. Similarly, the limiting rate of flow necessary for inhibition of growth or for loss of attachment increased generally with the initial age of the barnacles. Since observations were made at intervals during the experiment it was also possible to note the period of rotation necessary to bring about the first losses in attachment. This also increased generally with increasing age of initial growth. The observations are set forth in detail in Tables V and VI and summarized in Table VII.

In Table V the number of barnacles per square inch is recorded for various distances from the center of the disc, and for each individual sector. These observations are repeated for various intervals of time during the experiment.

An increase took place in numbers on the control panels due to continued attachment of cyprids, whereas on the rotated disc barnacles became progressively reduced in number as the experiment continued. After one hour of rotation the only losses were among the six hour old barnacles on Sector I. These losses were very small, however, and although observed at current velocities of 1.5 knots and 10 knots, they did not occur at intervening velocities. The first significant losses took place twelve hours after the start of rotation among barnacles less than twelve hours old. Of the older ones, only those 5 days old showed losses, amounting to a change at most velocities from 35 to 30 per unit area.

Barnacles on the remaining sectors, varying in age from twelve hours upwards, were not observed to diminish in number until the third day of rotation. The smallest loss over this period took place among the twelve hour barnacles, which were only lost at speeds of ten knots, whereas, after a similar period of rotation, other barnacles of greater ages had been lost even at 1.5 knot current velocity. Ten day old barnacles were not greatly disturbed by 1.5 knot currents until after eleven days' rotation. These observations, summarized in Table VII, indicate that in general the velocity of current necessary to dislodge barnacles increased with the age they had reached before the experiment. Barnacles twelve hours old, however, showed a marked increase in resistance to being dislodged, even compared to those ten days old, whereas those two days old, on the other hand, seemed to be less resistant than younger or older ones. It is also noteworthy that in general the duration of rotation necessary to bring about the first losses depends much more upon the initial age of the barnacle than upon the current velocities.

In a similar manner the percentage of the original number of barnacles remaining following an eleven day period of rotation increased with increasing age before rotation. The ten day old barnacles at the end of the experiment, however, showed poorer resistance and had a lower percentage of continued attachment than in the case of the five day barnacles. It must be noted, however, that the ten day barnacles were densely crowded and it is possible that this factor decreased their resistance to dislodgement.

Further examination of the results in Table V discloses that losses continued to occur to some extent on all sectors at the end of eleven days, although they were less on those sectors where initial age of the barnacle was greater.

Observations of the size of barnacles recorded during the experiment are given in Table VI. On Sector I the six hour old barnacles within a diameter of 8 inches, equivalent to a three knot water current, continued to grow, although at a slow rate.

## TABLE VI

*Growth of barnacles in relation to flow of water over the surface of a rotating disc, expressed as greatest diameter in millimeters* *
*(Figures in brackets refer to stationary control)*

| Stationary growth period | Diameter of disc (Inches) | Velocity (Knots) | Period of rotation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 hrs. | 1 hr. | 12 hrs. | 3 days | 5 days | 7 days | 9 days | 11 days |
| Sector I | Control | (0) | (0.3) | (0.3) | (0.3) | (0.5) | (0.8) | (1.3) | (2.5) | (3.0) |
| | 4 | 1.5 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.5 |
| | 8 | 3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| 6 hrs. | 12 | 5 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| | 16 | 6.5 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| | 20 | 8 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| | 24 | 10 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Sector II | Control | (0) | (0.3) | (0.3) | (0.3) | (0.6) | (0.8) | (2.5) | (3.0) | (3.5) |
| | 4 | 1.5 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 1.0 | 1.5 |
| | 8 | 3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| 12 hrs. | 12 | 5 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| | 16 | 6.5 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| | 20 | 8 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| | 24 | 10 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Sector III | Control | (0) | (0.3) | (0.3) | (0.3) | (0.6) | (0.8) | (2.0) | (3.0) | (3.5) |
| | 4 | 1.5 | 0.3 | 0.3 | 0.3 | 0.5 | 0.5 | 1.0 | 2.0 | 3.0 |
| | 8 | 3 | 0.3 | 0.3 | 0.3 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 1 day | 12 | 5 | 0.3 | 0.3 | 0.3 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| | 16 | 6.5 | 0.3 | 0.3 | 0.3 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| | 20 | 8 | 0.3 | 0.3 | 0.3 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| | 24 | 10 | 0.3 | 0.3 | 0.3 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Sector IV | Control | (0) | (0.3) | (0.3) | (0.5) | (0.8) | (1.0) | (3.0) | (3.5) | (4.0) |
| | 4 | 1.5 | 0.3 | 0.3 | 0.5 | 1.0 | 1.0 | 1.3 | 1.3 | 1.5 |
| | 8 | 3 | 0.3 | 0.3 | 0.3 | 0.8 | 0.8 | 0.8 | 0.8 | 1.0 |
| 2 days | 12 | 5 | 0.3 | 0.3 | 0.3 | 0.8 | 0.8 | 0.8 | 0.8 | 1.0 |
| | 16 | 6.5 | 0.3 | 0.3 | 0.3 | 0.3 | 0.8 | 0.8 | 0.8 | 0.8 |
| | 20 | 8 | 0.3 | 0.3 | 0.3 | 0.3 | 0.8 | 0.8 | 0.8 | 0.8 |
| | 24 | 10 | 0.3 | 0.3 | 0.3 | 0.3 | 0.8 | 0.8 | 0.8 | 0.8 |
| Sector V | Control | (0) | (0.8) | (1.0) | (1.5) | (2.5) | (3.0) | (4.0) | (5.0) | (6.0) |
| | 4 | 1.5 | 0.5 | 0.8 | 1.0 | 2.5 | 2.5 | 3.0 | 3.0 | 3.5 |
| | 8 | 3 | 0.5 | 0.8 | 0.8 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| 5 days | 12 | 5 | 0.5 | 0.5 | 0.8 | 1.5 | 1.5 | 1.0 | 1.5 | 1.5 |
| | 16 | 6.5 | 0.5 | 0.5 | 0.8 | 1.5 | 1.5 | 1.0 | 1.5 | 1.5 |
| | 20 | 8 | 0.5 | 0.5 | 0.8 | 1.5 | 1.5 | 1.0 | 1.5 | 1.5 |
| | 24 | 10 | 0.5 | 0.5 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 |
| Sector VI | Control | (0) | (2.5) | (2.0) | (2.5) | (4.0) | (5.0) | (7.0) | (9.0) | (9.0) |
| | 4 | 1.5 | 2.0 | 2.0 | 2.0 | 3.5 | 3.5 | 4.5 | 4.5 | 5.0 |
| | 8 | 3 | 2.0 | 2.0 | 2.5 | 3.0 | 3.0 | 3.5 | 3.5 | 3.5 |
| 10 days | 12 | 5 | 2.0 | 2.0 | 2.0 | 3.0 | 3.0 | 3.5 | 3.5 | 3.5 |
| | 16 | 6.5 | 2.0 | 2.0 | 2.0 | 3.0 | 3.0 | 3.5 | 3.5 | 3.5 |
| | 20 | 8 | 2.0 | 2.0 | 2.0 | 3.0 | 3.0 | 3.5 | 3.5 | 3.5 |
| | 24 | 10 | 2.0 | 2.0 | 2.0 | 3.0 | 3.0 | 3.5 | 3.5 | 3.5 |

* Experiment initiated 2/19/45.

TABLE VII

*Summary of observations on the effect of water currents produced by rotation upon attached barnacles. Expressed as multiples of normal growth, period before loss in number first occurred, and percentage remaining at the end of eleven days* *

| Current velocity (Knots) | Period of stationary growth | | | | | |
|---|---|---|---|---|---|---|
| | 6 Hrs. | 12 Hrs. | 1 Day | 2 Days | 5 Days | 10 Days |
| 1.5 | 0.1 × (1 Hour) 20% | 0.4 × 5 Days 30% | 0.8 × 3 Days 30% | 0.3 × 3 Days 50% | 0.6 × 3 Days 90% | 0.6 × 11 Days 70% |
| 3 | *Inhibition* 12 Hours 30% | *Inhibition* 5 Days 40% | *Inhibition* 3 Days 40% | 0.3 × 3 Days 60% | 0.2 × 2 Days 90% | 0.3 × 3 Days 40% |
| 5 | — 12 Hours 20% | — 5 Days 20% | — 3 Days 30% | 0.1 × 3 Days 30% | 0.2 × 2 Days 70% | 0.3 × 3 Days 40% |
| 6.5 | — 12 Hours 20% | — 5 Days 20% | — 3 Days 20% | *Inhibition* 3 Days 30% | 0.2 × 2 Days 60% | 0.3 × 3 Days 30% |
| 8 | — 12 Hours 20% | — 5 Days 20% | — 3 Days 20% | — 3 Days 20% | *Inhibition* 2 Days 60% | 0.3 × 3 Days 30% |
| 10 | — (1 Hour) 10% | — 3 Days 20% | — 3 Days 20% | — 3 Days 20% | — 2 Days 60% | 0.3 × 3 Days 30% |

* Experiment initiated 2/19/45.

Even at velocities below 1.5 knots the growth was considerably less than that of the stationary control. The ten day old barnacles on Sector VI continued to grow near the circumference, although subjected to ten knot currents. Barnacles of intermediate age showed a reduction of growth inversely dependent upon their initial age, and directly dependent upon the current. Barnacles less than two days old ceased growth at water currents over three knots, and only the ten day old barnacles continued growth at ten knots.

At the end of this experiment the remaining barnacles were examined to determine whether any considerable portion remaining attached was dead. A few of the older barnacles had lost their soft parts, but it was difficult to obtain a reliable criterion as to the condition of the remainder. It was not definitely established, therefore, what proportion had died.

Differences between the results of this and the previous experiment lie mainly in the higher current velocities required to bring about growth inhibition and loss of attachment and the absence of complete loss at any part of the disc in the later experiment. This apparent anomaly may be partly due to the much greater density of initial attachment, which may have reduced the *effective* velocity of the currents by providing a much more irregular surface. It is also conceivable that had the

experiment continued for the full 19 day period of the first experiment rather than 11 days only, the growth inhibition effect might have appeared at lower velocities and might have resulted in complete loss at some parts of the disc.

## Discussion

The experimental results indicated that current velocities limiting the attachment of barnacles lie in the neighborhood of one knot and that the limits for the three species, *Balanus eburneus, B. amphitrite,* and *B. improvisus* are in ascending order of magnitude. That a one knot current should limit attachment is in accordance with the conclusions of Visscher and earlier workers that attachment of barnacles does not readily take place upon ships in motion.

It further appears that growth rates of previously attached barnacles are reduced by water currents in inverse proportion to the age of the barnacle and directly in proportion to the current velocity. The effect of crowding together is further to reduce the growth rate. The effect of very low current velocities is to increase the rate of growth. A slight loss of attachment occurs at all current velocities, but this may be partly due to overcrowding. Large and significant losses take place at velocities which are sufficient to reduce the growth rate. These losses begin some hours after the start of rotation, and continue for a period of at least eleven days.

Since losses due to dislodgement do not occur during the first few hours of rotation, except in the case of barnacles attached within six hours or less, and since they continue to take place during the entire period of rotation, it seems probable that they are not directly due to mechanical action. Possibly the mechanism is one of interference with feeding processes, followed by reduced growth rate, death and diminished adhesion.

A further point arising out of the experimental data is the fact that at equivalent current velocities 12 hour and 24 hour old barnacles show greater growth and less loss of attachment than those somewhat older. It is possible that at this stage of development, with metamorphosis incomplete, an orientation to the current may occur which facilitates feeding, although this was not actually observed.

Once attached the barnacles are able to withstand current velocities of increasing magnitude, although for the first two or three weeks the growth rate is considerably reduced. Loss of attachment appears to follow reduced growth rate and may involve actual death from lack of food. The current velocities required to bring this about are sufficiently low to provide support for Visscher's conclusion that barnacles are killed by ocean passages of 500 miles or more, if vessels of more than 10 knots performance are considered, or if the age of attached barnacles is no greater than a 10 day stay in port would allow. On the other hand, current velocities lying below this figure, such as would be produced by slow sailing vessels, would appear from the experimental data to be insufficient to bring about a loss of the attached barnacles. In the case of faster ships, stays in port of longer than a few days might also permit the accumulated barnacles to survive an ocean passage. This type of ship service would readily provide a means of distribution for barnacles. It would explain Fischer-Piette's observations of the appearance of *Balanus amphitrite* on Mediterranian shores, and would support his hypothesis of distribution by ships.

The enhanced growth rate in current velocities of 1.3 knots and less, depending on the age of the barnacle, provides a quantitative expression for the observations

of Rice, Moore, Kitching, and others upon the increased growth of barnacles subjected to water currents.

The conditions in a harbor or an embayed or open coast are rarely such that tidal currents in excess of one knot are continuously present. Under these conditions, therefore, there would be no obstacle to the attachment of barnacles. Once attached, however, the barnacles would usually be subjected to intermittent tidal currents.

No data are available as to the effects of intermittent currents but it would be reasonable to assume that these would be, if anything, less than the effects of continuous currents. The data presented here would therefore make it appear unlikely that any but the strongest of tidal currents would prevent the attachment and continued development of barnacles on rocks or stone, and wooden piling. Where fairly continuous currents of less than 1.5 knots were present the growth would be encouraged.

A different situation occurs in estuaries where continuous currents might occur. Here, however, the limiting factor, according to the work of previous authors, is probably lowered salinity, since continuous currents would scarcely permit of the estuary remaining salt.

Where wave action is strong the effects might be considered as equivalent to strong currents of frequently changing direction, which might conceivably inhibit attachment or growth, even below tide levels. Even where attachment occurred during temporary lulls, the strong recurrent wave action might be expected to dislodge the barnacles. This may explain the observations of Stephenson and his co-workers that some species of barnacles do not occur in a zone of heavy wave action.

The effect of water currents under natural conditions may therefore be summarized as one of enhanced growth with velocities below 1.5 knots, and one of reduced growth above this. It would rarely happen under natural conditions that water currents alone would prevent barnacle colonization. Strong wave action, however, might be expected to have this effect.

Certain qualifications should be added to the above discussion which may explain some anomalous observations. The arguments above are based on the calculated rates of flow of water over the rotating disc, and the results of experiments have indicated that these rates are slightly higher than the actual rates of flow taking place. The error, however, does not appear to be greater than 10 per cent to 20 per cent, and does not qualitatively affect the validity of the general conclusions.

Since the observations were made upon the relatively smooth surface of a wooden disc, they would not be applicable to very rough surfaces where local pockets of relatively still water might develop, to the areas immediately adjacent to lapped plates of ships' hulls, or to portions of piling or other submerged surfaces where the contours and configuration might produce local stagnation effects.

## Summary

1. The work of previous authors, dealing with the effect of water currents upon barnacle attachment, growth and distribution is briefly reviewed.

2. Experiments were conducted to determine the effects of water currents upon the attachment and growth of barnacles, and particularly of *Balanus amphitrite*.

Submerged rotating discs and glass tubes of graded cross-sectional diameter were employed to provide variations in current velocity.

3. The velocity of water current limiting attachment appears to lie between 0.5 and 0.9 knot for *Balanus amphitrite,* between 0.4 and 0.7 knot for *B. eburneus,* and above 1.1 knots for *B. improvisus.*

4. Following attachment the growth rate of barnacles was found to be increased by water currents of velocity less than 1.5 knots and to be decreased by currents with velocities in excess of this. The adverse effects of water currents were found to decrease with increasing age of the barnacles subsequent to attachment. Six hours after attachment, growth rate was reduced to one-third of normal by a 1.5 knot current and completely stopped by a 3 knot current. Five days after attachment, growth was prevented by currents ranging between 3.3 and 8 knots.

Loss of attachment appeared to some extent among all barnacles in which growth rate was reduced. This loss was greatest at velocities bringing about complete cessation of growth.

5. It is suggested that loss of attachment is due to interference with the feeding process, followed by reduction of growth rate, death, and diminished adherence. Possibly due to an orientation to the current which facilitates feeding, barnacles attached for one day or less show less retardation of growth rate and loss of adherence than barnacles two days old.

6. Since tidal currents are almost invariably intermittent it appears from the data presented that they are not sufficient to prevent the colonization of suitable surfaces by barnacles, except where the velocities are unusually high.

It also follows from the numerical results obtained that on vessels making short stays in port and relatively long voyages, little permanent barnacle fouling will occur, since those organisms which attach will be killed and at least a portion of them dislodged. The evidence does not preclude, however, the continued growth of barnacles upon slow vessels making longer stays in port, and their geographical distribution by this means.

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# MICRURGICAL STUDIES ON CHIRONOMUS SALIVARY GLAND CHROMOSOMES [1]

ETHEL GLANCY D'ANGELO

*Department of Biology, Washington Square College, New York University, and Queens College,[2] Flushing, New York*

Particular interest in the giant salivary gland chromosomes of dipteran larvae was awakened by Painter (1933) and Heitz and Bauer (1933) who demonstrated the existence of a close relationship between the genetic significance of the chromosomes and their structure. Regarding the gross structure of these chromosomes there is general agreement, but not for fine details. Bauer (1936) and Painter and Griffen (1937) offered cytological evidence for a polytene concept of chromosome structure from observation, in fixed and stained preparations, of numerous longitudinal striations which they considered to be chromonemata. Metz and Lawrence (1937) and Metz (1941) maintained that the longitudinal striations appearing in these fixed and stained preparations were merely the result of drawing out the walls of alveoli. Buck (1942) afforded support for this view by showing in osmic vapor-treated chromosomes that an original alveolar structure could be reversibly transformed into a fibrillar-like one by micromanipulation. Kodani (1942), using cytolytic methods with alkali and urea, found evidence for only four chromonemata in each chromosome, a condition which Painter and Griffen (1937) and Buck (1937) observed in the early larval stages, but not in the giant chromosomes of later stages. More recently, Frolova (1944) has given experimental support to the polytene concept by demonstrating the presence of numerous longitudinal strands in fixed chromosomes after partial digestion with nuclease preparations.

Aside from their cytogenetic interest, the salivary gland chromosomes, because of their large size and visibility in vivo, make especially suitable material for studies on physical properties. Using the micrurgical technique, various investigators (Vonwiller and Audova, 1933; Barigozzi, 1938; Stefanelli, 1939; and Pfeiffer, 1940) have found the freshly isolated salivary gland chromosomes to be tough, viscid, elastic gels. These observations are in essential agreement with those first made by Chambers and Sands (1923) on the chromosomes of Tradescentia pollen mother cells and by Chambers (1924a) on those of Dissosteria spermatocytes. Buck (1942), using osmic vapor-treated chromosomes, reported more detailed findings on elasticity. That the physical properties of the chromosomes may be easily modified by the presence of torn cytoplasm or calcium ions was shown by Duryee (1941) in his studies on amphibian chromosomes. The observations of Chambers (1924b) that torn cytoplasm is acidic, and of Chambers and Reznikoff (1926) that the micro-injection of various salts into protoplasm may have detrimental effects,

[1] This work, done at Washington Square College, New York University, and at the Marine Biological Laboratory, Woods Hole, Mass., is presented in partial fulfilment of the requirements for the degree of doctor of philosophy at New York University.

[2] Present address.

further point out the need for precaution in studying the physical properties and structure of the chromosomes.

This investigation is concerned with the structure and physical properties of the salivary gland chromosomes as observed within the intact fresh cell and after isolation into media designed to maintain them in the fresh condition.

Grateful acknowledgment is made to Prof. Robert Chambers, under whose guidance this work was done, for his constant aid and invaluable criticism.

## Materials and Methods

The species selected for study were *Chironomus plumosus, C. tentans,* and certain smaller unidentified species of Chironomus. Chironomus larvae were chosen in preference to those of other Diptera because of their larger chromosomes (maximum size, 20 $\mu$ in diameter and 150 $\mu$ long) and the flattened shape of the salivary gland, factors which facilitate micromanipulation. Moreover, the chromosomes were always plainly visible in the living cell, the bands being especially well defined. Incidental observations were made on Drosophila chromosomes and they were found to agree in all important respects.

The glands were carefully removed from mature larvae under a dissecting microscope and immediately immersed in either a drop of Chironomus hemolymph or amphibian Ringer's solution which was found to be isotonic. Care was taken to avoid the presence of any tissue but the gland itself since otherwise the medium became unduly acid. The hemolymph was obtained by inserting a pipette through the body wall of a larva into the hemocoele. In order to be satisfactory the hemolymph had to be freshly obtained and immediately covered with paraffin oil to prevent the development of alkalinity which occurred on exposure to air.

The micromanipulation was carried out immediately after mounting a single intact gland in the medium on a thoroughly cleaned coverslip which was then inverted over the moist chamber of a Chambers micromanipulator. Structural changes within the chromosomes of the intact cells do not appear for many hours in hemolymph or Ringer's solution, but extra precautions were taken by making observations within 15 minutes after mounting the glands. The micropipettes routinely used had an inside bore of never over one $\mu$, and the microneedles used for fine dissection of the chromosomes tapered rapidly to points approaching the limit of visibility.

Nuclei and chromosomes were removed from disintegrating cytoplasm as quickly as possible. This was necessary in order to preserve their normal appearance, an observation in agreement with that of Duryee (1937) on amphibian germinal vesicles. To remove the chromosomes, an entire gland was mounted under a dissecting microscope in a medium, to be described later, in which the chromosomes were to be isolated. By means of fine steel needles a cell was quickly torn and the nucleus ruptured. The torn nucleus remained in situ, and, by exerting a gentle pressure on the torn cell, the chromosomes could be forced out of the nucleus and floated away without coming in contact with the injured cytoplasm. The loosely adhering clump of chromosomes was immediately transferred by means of a lip pipette to a fresh drop of the medium to be used, then mounted in the usual manner in the moist chamber. In this way the chromosomes were found to retain most nearly their normal appearance and physical properties after isolation, their sub-

sequent condition depending on the medium in which they remained. No medium was found which would maintain the chromosomes in their normal condition indefinitely nor were extensive attempts made to find such a medium. Among the media tried and found to be unsatisfactory were hemolymph, Ringer's solution, paraffin oil, 0.1 M NaCl–0.01 M KCl solution used by Duryee (1941), 0.25 per cent egg albumen solution used by Melland (1938), and 0.3 M sucrose solution suggested by the work of Chambers and Sands (1933). These special solutions were used without regard to pH conditions.

From determinations on a great variety of cells including the Chironomus salivary gland nucleus, Chambers (1929) found the intranuclear pH to be within 7.6–7.8. It would be expected therefore that the medium in which the isolation of the chromosomes is to be done should have a pH within the aforementioned limits. However, the most satisfactory mixture, when buffered to pH 7.6–7.8, caused the isolated chromosomes to swell and become excessively sticky. Moreover, the swelling was accompanied by a fading of internal structure. However, at pH 7.0, the chromosomes closely approached, both in appearance and physical properties, those within the nucleus. It should be noted that the consistency was slightly greater than that of the intact chromosomes. The structure and properties of the chromosomes were maintained throughout the experimental period. The medium used consisted of 0.09 M KCl, 0.06 M NaCl, and 0.005 M phosphate buffer, pH 7.0.

Observations and micromanipulation experiments were routinely made with a Leitz microscope using a 1.8 mm. oil immersion objective, N.A. 1.25, and a 10 × ocular. A micrometer ocular was used for measurements.

## Observations

### *The chromosomes in the fresh cell in Ringer's solution or hemolymph*

The chromosomes and nucleolus in the fresh cell were always plainly visible, the bands being sharply defined and either beaded or homogeneous in appearance. A hyaline material, variously referred to as nuclear sap, nucleoplasm, or karyolymph, separated the chromosomes from each other. It has been shown (Glancy, 1940) that this hyaline material is differentiated into a central portion of jelly-like consistency in which the chromosomes are embedded, and a more fluid peripheral zone. The evidence for this regional differentiation was based on experiments involving the injecting of oil drops and carbon particles as well as the manipulation of the chromosomes. The hyaline material is referred to in this paper as the nuclear matrix.

**Stickiness.** It was found possible to insert microneedles into the nucleus and to force the chromosomes against each other as well as against other structures in the nucleus, and also to push them against injected oil drops and carbon particles. Two chromosomes pushed together by means of microneedles could easily be separated again without sticking to each other. It should be noted, however, that a narrow hyaline zone, presumably the jellied nuclear matrix, always remained between them, thus probably preventing actual contact of the two chromosomes. Similar results were obtained when a chromosome was forced against the nucleolus or the nuclear membrane.

A micropipette containing an aqueous carbon suspension was inserted as close as possible to a chromosome without actually touching it, and a small quantity of the suspension injected. In none of the attempts were carbon particles seen to adhere to the chromosomes, nor did they ever make contact with a chromosome. However, injection of a large amount of fluid caused liquefaction of the jelly matrix, thus permitting the carbon particles to make contact with the chromosomes. In a similar manner, various oils; namely, Nujol, olive, almond, and peanut oils, were injected into the nucleus. None of the oil drops adhered to the chromosomes.

When the cytoplasm was torn without tearing the nucleus, a change occurred in the nucleus. This was made evident by the marked shrinkage of the chromosomes. Two chromosomes pushed together in the nucleus of such a torn cell invariably stuck to each other, and thick viscid strands were pulled out when attempts were made to separate them. The chromosomes also adhered to the nucleolus, the nuclear membrane, injected oil drops, carbon particles, and the microneedles. In every case, separation could be achieved only when accompanied by thick viscid strands pulled out from the chromosomes.

In summary, these experiments show that the chromosomes, because they are prevented from coming in contact by the jelly-like nuclear matrix, appear to be nonsticky in the intact cell. Liquefaction of this matrix occurs when the cytoplasm is torn, thus permitting the chromosomes to make contact. They are then found to be sticky.

**Consistency.** The micrurgical experiments to be described indicate that, compared to the cytoplasm and the nuclear matrix, the chromosomes are relatively soft, easily deformable gels, and that there are differences in consistency between the bands and the interband regions.

A very finely tapered microneedle was pushed up through the cytoplasm and through the nuclear membrane, into the interior of the nucleus. The needle was pushed in from the side, and even before it was brought into actual contact with a chromosome, the surface of the chromosome became indented on the side next to the needle. The needle was pushed still farther and when its tip went into the interior of the chromosome, the indentation disappeared and the original contour of the chromosome was restored. This deformation of the chromosome must be due to the central jelly-like matrix since insertion of the needle into the more fluid peripheral region of the nucleus had no such deforming action on the chromosomes. The indentation of the chromosome, when the needle tip was being brought into its vicinity, and the readiness with which the chromosome returned to its original shape after the needle had penetrated indicated that the chromosome was of a softer consistency than the surrounding central nuclear matrix.

The relatively low consistency of the chromosomes compared to the central nuclear matrix was also shown by experiments in which oil drops were injected into the nucleus. The effect of the oil drops in causing deformation of an adjacent chromosome differed according to the site of injection. Drops of Nujol, olive, almond, and peanut oils, 1–20 $\mu$ in diameter, were injected close to a chromosome. Large oil drops carefully injected into the central jelly-like nuclear matrix produced a rounded depression in the nearest chromosome. However, this did not occur when the matrix was agitated by the needle, thus showing the thixotropic nature of the jelly matrix. Peripherally injected oil drops were never observed to cause de-

formation. These results indicate that the consistency of the central nuclear matrix is greater than that of the chromosomes, whereas the reverse is true in the peripheral region of the nucleus.

The nucleolus was found to be the most easily deformed body in the nucleus. This was shown by forcing the nucleolus against the nuclear membrane, the chromosomes, and the microneedles. In all cases the nucleolus was deformed in the manner of a fluid drop. When the nucleolus was separated by a microneedle from the small fourth chromosome to which it is normally attached in Chironomus, the nucleolus became spherical.

Evidence for differences in consistency between the bands and the interband regions was obtained in several ways. A microneedle inserted into a chromosome and then withdrawn produced a space which closed over more rapidly in an interband region than in a band. The thin bands were intermediate between the interband regions and the thick bands in this respect. The longer persistence of the hole in the bands is indicative of a higher consistency. The greater resistance of the bands, especially the thicker ones, to deformation during manipulation of the chromosomes, as in the stretching experiments, also is in accord with their higher consistency. The results of injecting oil drops into the chromosomes led to similar conclusions. Of the various oils used, Nujol, olive, almond, and peanut oils, only the last two could be readily injected into a chromosome. This was easily done in an interband region, but only with difficulty or not at all in a band. The injected oil drops became spherical, showing the relatively low consistency of the interband regions, and caused the displacement of the relatively viscid bands on either side.

**Extensibility and elasticity.** Observations on extensibility and elasticity were made by stretching a small portion of a chromosome between two microneedles. The degree of stretching which could be performed, however, was limited since the nuclear membrane was torn when the microneedles were moved more than approximately 20–30 $\mu$ apart. In all cases in which a part of a chromosome was stretched within the nucleus, release from the needles resulted in a complete return to the original appearance.

A short region of a chromosome was seized lengthwise between two microneedles and the needles were slowly drawn apart. As the chromosome was stretched, there was a decrease in its diameter, and its structure became less distinct. The thick bands were more resistant to stretching than the thinner ones, whereas the interband regions stretched most readily. During the stretching the broad bands often separated into several narrow bands, thus demonstrating their compound nature. Faint longitudinal striations which followed the lines of tension generally appeared in the interband regions when a chromosome was stretched more than twice its length. These stress lines became more pronounced as stretching was increased. They did not appear to be identical with the delicate longitudinal striations occasionally seen in unstretched fresh chromosomes. On release of tension, the striations disappeared and the chromosome, even after being stretched five times its length, returned to its original condition. It was not found possible to stretch a given region more than this without tearing the nuclear membrane. In every case, the chromosomes recovered completely after the microneedles were removed.

Lateral stretching of the chromosomes was also performed. Two microneedles were inserted into a chromosome close to the margins, then slowly drawn apart.

The chromosome cylinder flattened during the process and bands which had appeared homogeneous became beaded (Fig. 1). As tension was increased, stress lines, radiating from the point of each microneedle to the chromosome, became visible. On release of tension these lines disappeared, and the beaded appearance of the bands was lost. A chromosome could be stretched approximately three times its original width and recover fully.

a b

FIGURE 1a. Sketch of a portion of a chromosome showing two microneedles inserted directly into a band prior to lateral stretching of the chromosome within the intact cell. All sketches are of Chironomus chromosomes.

FIGURE 1b. Beaded appearance of the band when the chromosome in (a) is stretched laterally. Note tension lines in the interband regions.

**The chromosome membrane.** The micrurgical evidence to be presented indicates that the salivary chromosome possesses a delicate elastic membrane. A microneedle was inserted into the margin of a chromosome within the intact nucleus, in an attempt to remove any existing membrane. When the needle was slowly and carefully withdrawn, faint lines were seen radiating from it to the chromosome (Fig. 2a). To test whether or not these lines were actually folds in

a b c

FIGURE 2a. Lifting by a microneedle of a membrane-like material from a portion of a chromosome within the intact cell.

FIGURE 2b. Micro-injection of a carbon suspension into the area subjacent to the lifted membrane shown in (a).

FIGURE 2c. Concentration of the carbon particles into a narrow zone as a result of gradual contraction of the membrane.

a membrane which had been lifted off from the chromosome the same operation was repeated and an aqueous carbon suspension was injected into the triangular area in question. The carbon particles converted the region showing the radiating lines into a sac-like structure which remained connected to the chromosome (Fig. 2b). When the micropipette was carefully withdrawn, the fluid evidently escaped from the area, as the sac slowly contracted and the carbon particles came closer to the chromosome where they became packed against its surface (Fig. 2c). Repetition of the

experiment injecting a larger quantity of the suspension into the triangular area first converted it into a sac; then as injection was continued, the sac burst, and the carbon particles scattered in the matrix where they briefly exhibited Brownian movement.

Oil drops were also injected in a similar manner into the triangular area produced by pulling on the margin of the chromosome. After the withdrawal of the micropipette, the oil was gradually drawn to the chromosome surface where it remained as a slight protuberance. Larger oil drops were injected and could be seen to be held against the chromosome by what appeared to be a membrane. No deformation of the chromosome occurred, presumably because there was no jelly-like matrix between the oil drop and the chromosome.

Various salt solutions (0.01–1.0 M NaCl, KCl, and $CaCl_2$) and also distilled water were injected into an interband region in an amount which caused a swelling of the region without breaking its contour. Figures 3a, b, and c show the condi-

FIGURE 3a. Micropipette inserted into an interband region of a portion of a chromosome within the intact cell showing the initial stage of injection.

FIGURE 3b. Localized swelling and loss of structure in the injected portion of the chromosome. Results are similar with solutions of NaCl, KCl, $CaCl_2$, and distilled water.

FIGURE 3c. The appearance of the same chromosome after injection of sufficient $CaCl_2$ solution to rupture the injected region. The bands have reappeared in their original positions, but the distinct boundary (membrane) shown in (a) is no longer evident. Note the granular region of the nuclear matrix.

tion of the chromosome before, during, and after the injection. With the progress of the injection, the lesser bands in the vicinity faded out and the zone underwent swelling until a heavy band on each side of the injected zone was reached. The injection was continued within the limit of bursting. The pipette was then removed, and the swollen zone returned to its original dimensions in which the final appearance was that of the region prior to injection. The surface of the injected region could be ruptured when the amount of fluid injected was sufficient to cause it to swell to approximately two and a half times its original diameter. Thereupon the membrane-like boundary suddenly disappeared irrespective of the fluid injected. When $CaCl_2$ was used a granulation appeared in the nuclear matrix around the injected zone (Fig. 3c). This did not occur with water, NaCl, or KCl solutions.

It may be concluded, therefore, that the salivary chromosome possesses a delicate elastic membrane.

**Internal Structure.** The visible structure of the chromosomes was studied by careful and extended observations of the chromosomes within the intact nucleus

both of the isolated gland and of glands within the intact larvae.[2] For this purpose the observations were made with a Leitz 1.4 N.A. apochromatic oil objective and a triple lens condenser. The equipment was sufficient to resolve the hexagonal apertures of the *Pleurosigma angulatum*. The distinct and sharply defined bands were beaded in some regions, and homogeneous in appearance in others. The interband regions, which were much less refractile, generally appeared either homogeneous or finely granular. In unusually large chromosomes the granulation was coarse and often the arrangement gave the effect of longitudinal striations. These granular striations, which were seen at all levels in the chromosomes, were individually connected to the beads in the bands. During these observations the chromosomes were not under tension and in the case of the isolated gland were free from compression.

That the salivary gland chromosome is made up of numerous longitudinal fibrils was further indicated by microdissection. The chromosome membrane was first removed by a very fine microneedle over a length of several bands from the region to be studied. The needle was then inserted into the margin of an exposed interband region. This adhered to the needle and, when the needle was carefully moved to one side, a strip of very thin fibril with one end on the tip of the needle could be seen being carried away from the chromosome. As the fibril was being carried still farther it was separated from the chromosome over a length of several bands (Fig. 4).

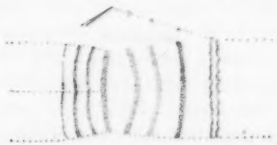

FIGURE 4. A single fibril with a chromonema-like appearance being removed by a microneedle from a chromosome within the intact cell.

The isolated fibril showed nodular swellings which corresponded in position with the bands from which it was being separated. This was done over and over again with the same chromosome and with different chromosomes. The same phenomenon could be observed whether the bands on the operated chromosome were homogeneous or beaded. The fibrils could be stretched and, when tension was released, they returned to their original lengths. One case was observed in which a fraying of the chromosome resulted when a pipette had been inserted to inject water. The injection was made close to the periphery and the pipette then removed. These partially isolated fibrils closely resembled that in Figure 4. The microdissection experiments were performed more readily with large chromosomes in which longitudinal striations were visible. They were also done with the chromosomes in which the interband regions appeared homogeneous. The dissection evidence strongly suggests that longitudinal fibrils possessing nodular swellings really exist in the chromosomes.

**The state of the chromosomes in nuclei rendered optically homogeneous by alkaline or hypotonic Ringer's solution.** The question has frequently been

[2] As Buck (1938) has already described, the intact larvae can be mounted between slide and coverslip and because of the transparency of its body wall, the cells in the salivary glands are easily visible under high power, and can be satisfactorily observed. Their appearance was identical with that of the isolated glands mounted in Ringer's solution.

raised whether chromosomes in so-called hyaline nuclei maintain their morphological integrity or whether they lose their identity through dispersion in the hyaline phase. Naturally occurring hyaline salivary gland nuclei were never found, but could be readily produced by immersion of the gland in slightly hypotonic or in mildly alkaline (pH 8.4) Ringer's solution. Within a few minutes after immersion the nucleus swelled, chromosomes faded, the interbands disappearing first, and finally only the nucleolus remained visible. When the gland was returned after several minutes to isotonic Ringer's solution at pH 7.0, the chromosomes reappeared.

A microneedle could be inserted into one of these hyaline nuclei without causing its collapse and usually without producing any visible effect. In some cases, however, the nuclear membrane wrinkled slightly when the microneedle was withdrawn, indicating that fluid was lost. When a microneedle was moved slowly back and forth within the nucleus no regional resistance to its movement was encountered. Similarly no resistance was offered to movement of the nucleolus throughout the entire nucleus. Return of the gland to Ringer's solution caused the chromosomes to reappear. It is noteworthy that the chromosomes which reappeared were frequently distorted in shape.

Carbon particles injected into the center of an untreated nucleus remained at the site of injection presumably because they were caught in the central jelly matrix in which the chromosomes are embedded. However, an injection made just under the nuclear membrane of an untreated nucleus, where the nuclear matrix is relatively fluid, resulted in a scattering of the carbon particles between and around the parts of the chromosomes in the peripheral region. None of the particles penetrated the central region of the nucleus. Exposure of such an injected nucleus to an alkaline or hypotonic Ringer's solution or to distilled water, caused the chromosomes to disappear. The fact that the chromosomes still retained their identity and did not go into solution was shown by the alignment of the carbon particles which kept their relative positions outlining the contiguous borders of what must be the swollen chromosomes (Fig. 5). The carbon particles failed to become completely dispersed

FIGURE 5. Alignment of carbon particles outlining the contiguous borders of adjacent chromosomes in an optically homogeneous nucleus. The nucleus was first injected with a carbon suspension, then hyalinized by immersion of the gland in hypotonic Ringer's solution.

as would be expected if the chromosomes had gone into solution. In nuclei which were first made hyaline, then injected with the carbon suspension in the peripheral region, a similar alignment of the carbon particles resulted. In both cases the chromosomes reappeared in the expected positions when the glands were placed in Ringer's solution.

It may be concluded, therefore, that although moving microneedles back and forth within the hyaline nucleus fails to reveal the presence of the swollen chromosomes, nevertheless the chromosomes have maintained their morphological integrity as shown by the injection of carbon particles.

*Isolated chromosomes*

The physical properties of the isolated chromosomes may be seriously affected by the methods used in their isolation and by the medium in which they are maintained. In the following experiments, attempts were made to maintain the properties which had previously been found for the chromosomes within the nucleus. It was found that rapid isolation of the chromosomes without contact with the torn cytoplasm was essential to prevent changes in structure and physical properties. Chromosomes which remained in the torn cell or came in contact with the cytoplasm during the isolation were shrunken, often distorted, and highly sticky. Of the various media tried, the one which caused the least change in the chromosomes after their isolation was the neutral KCl–NaCl solution described in the section on methods. In the experiments which follow on the isolated chromosomes, it is understood, unless otherwise stated, that the chromosomes were isolated into the neutral KCl–NaCl solution without coming in contact with the torn cytoplasm, transferred to a fresh drop of the same solution, and then manipulated. The duration of an experiment was not more than 10 minutes.

**Stickiness.** Chromosomes isolated into the neutral KCl-NaCl solution. Ringer's solution or hemolymph, were not unduly sticky. It was found that alkalinizing the medium (pH 8.0) caused the chromosomes to become sticky, whereas acidifying the medium (pH 6.5) reduced stickiness.

**Consistency.** The consistency of the isolated chromosomes in slightly alkaline KCl–NaCl solutions, pH 7.6–7.8, was found to resemble most closely that of the chromosomes in the intact cell, but structure was indistinct and the chromosomes were sticky. As the pH was decreased, the chromosomes shrank and the consistency increased. In neutral solutions little shrinkage occurred and the consistency was but slightly greater than that of the chromosomes within the intact cell. In acidic solutions (pH 6.0–6.5), the effects were more marked. At corresponding pH values, the consistency was always somewhat greater in Ringer's solution and in hemolymph than in the KCl–NaCl medium.

Chromosomes maintained in the neutral KCl–NaCl solutions were, like those in the intact nucleus, soft easily deformable gels which could be compressed without buckling. When they were cut or pulled apart by microneedles the cut or broken ends assumed smooth, rounded contours. This is in marked contrast to the results obtained with chromosomes which have been isolated after exposure of the glands to osmic acid vapors, formaldehyde, or acetic acid, following the methods of Buck (1942). Even brief treatment has a pronounced effect on the consistency of the chromosomes, causing them to become relatively rigid bodies which break squarely across when stretched.

It may be concluded that, although the consistency of isolated chromosomes maintained in a slightly alkaline KCl–NaCl solution most closely resembles that of the chromosomes within the cell, a neutral medium is preferable for manipulation since structure is still maintained and stickiness is lessened.

**Extensibility and elasticity.** The use of isolated chromosomes permits unrestricted stretching which obviously is impossible in the intact cell. It was found possible to stretch an isolated chromosome 10 times its original length and obtain complete recovery on release of tension. During the process of stretching, the interbands stretched somewhat more readily and so underwent a greater decrease in diameter than the bands. The wide bands separated into two or more narrow bands as occurred in the intact cell operations. As stretching was increased the diameter of the chromosome decreased so that at 10 fold elongation it appeared to be a mere thread with occasional nodular swellings. If this degree of stretching was not repeated more than 2 or 3 times, it was completely reversible. Repeated stretching caused permanent deformation, particularly of the interband regions, which remained in a partially stretched condition even after tension was released. Much greater stretching was possible without causing a chromosome to break in two. In some cases, a chromosome could not be broken within the limits of stretching of the micromanipulator. Thus one chromosome was stretched 25 fold without breaking. When tension was released, it returned to 1.3 × its original length. Other chromosomes broke in two when stretched 12 fold. The break always occurred in an interband region, although this was not apparent until the chromosome had partially recovered. Fibrillae were occasionally seen at the broken ends. It should be pointed out that greatly stretched chromosomes developed a coarse fibrillar structure and rapidly deteriorated. This fibrillar appearance was different from that observed in unstretched chromosomes in that these fibrils were relatively coarse, and always followed the lines of tension. Deterioration also occurred, although much more slowly, in chromosomes which were not manipulated, indicating that the medium is not all that is to be desired.

It was found that on the addition of acids (pH 6.0–6.5) or calcium ions to the KCl–NaCl medium, the consistency of the bands was increased more than that of the interband regions. This effect was especially marked in the acid solutions, the bands becoming curved or bent rather than stretching or separating into narrow bands when placed under tension. The interband regions, although having a higher consistency than in neutral solutions, nevertheless were readily extensible, and when stretched more than twice their length appeared coarsely fibrous. At a 5–10 fold elongation, a chromosome broke in an interband region as before with fibrillae being evident at the broken ends. In slightly alkaline solutions, pH 8.0, the chromosomes became extremely ductile and showed almost no elasticity. The loss of elasticity was partially prevented by the addition of calcium salts to the alkaline solutions.

Isolated chromosomes were also stretched laterally. These gave results qualitatively similar to those observed in the intact cell. The isolated chromosomes, however, could not be stretched more than approximately double their width without deteriorating. In some instances, at least, the deterioration was associated with tearing the chromosome membrane.

In some of the experiments Buck's (1942) method was used of exposing glands to osmic acid vapors for 18–24 hours at 5° C., then isolating the chromosomes in distilled water. The chromosomes could be isolated readily by this method, and, in agreement with Buck, were tough, relatively rigid bodies, breaking squarely across in an interband region when stretched more than four times their length. Occasional fibrillae appeared extending from the broken ends of the chromosomes. This

was also observed by Buck, although he attached no particular significance to it. Stretching was reversible only up to 2 fold elongation (or 100 per cent as stated by Buck). Thus these chromosomes were far less extensible and elastic than those in the fresh state.

**Structure.** The chromosomes isolated in the KCl–NaCl solution at pH 7.0 generally showed no structure either in the interband regions or in the bands. Occasionally fibrillae were visible in the interband regions. On the other hand, in an acid solution (pH 6.5), fibrillar structure was more generally evident. In those cases where the fibrillar structure was already apparent, it became more distinct when the medium was slightly acidified.

Isolated chromosomes were dissected in the same manner as the chromosomes in the intact cell. The fibrils removed from the isolated chromosomes, behaved, except for a slightly increased stiffness, similarly to those removed from the chromosomes within the nucleus.

Occasionally it was found possible to produce a breakdown of the chromosomes into numerous longitudinal fibrils. This was done as follows: an intact gland was immersed in a hanging drop of neutral KCl–NaCl or in amphibian Ringer's solution, then a large pipette, about 15 $\mu$ in diameter, was inserted into the nucleus of an intact cell and a single chromosome withdrawn. This could be done readily, indicating the ease with which the nuclear matrix becomes solated, and the relative non-adhesiveness of the chromosomes to each other. The chromosome was then expelled into the drop to one side of the gland, whereupon it sometimes became shredded into numerous longitudinal fibrils which resembled the fibrils obtained by dissection of an intact chromosome. This operation was successful only if the bore of the pipette was close to the diameter of the chromosome, thus suggesting the possibility that the chromosome membrane may have been removed while the chromosome was being either sucked into or expelled from the pipette. If the pipette was much larger than the chromosome, then the chromosome was expelled intact. The fibrils of the shredded chromosome were very numerous, sometimes appearing as a jumbled mass, and sometimes as a bundle of essentially parallel longitudinal fibrils. This experiment was repeated several times on the same day and on different days. The operation was not successful every time but the fault could be ascribed to the critical bore of the pipette and the speed with which ejection was made. The fraying of these chromosomes occurred irrespective of whether the medium into which they were expelled was NaCl–KCl or amphibian Ringer's solution. The operation was not done a sufficient number of times to discover an optimum medium for the fraying. The fact that it can be made to occur even very occasionally is highly significant.

## Discussion

The results of these micrurgical experiments indicate that the normal structure and physical properties of salivary gland chromosomes can be maintained only if due precaution is taken to avoid the deleterious effects of torn cytoplasm. The "acid of injury" reaction of torn cytoplasm, originally described by Chambers (1924b), is significant in this regard. More recently, Duryee (1941) has reported the injurious effects of torn cytoplasm and calcium ions on the isolated chromosomes and germinal vesicles of amphibian oocytes. The changes in structure and physical

properties of the salivary gland chromosomes which occur in a torn cell appear to be similar to those effected by acid or calcium ions. This suggests that calcium and hydrogen ions may be released from the injured cytoplasm.

The general finding that chromosomes isolated in media of a physiological pH are sticky raises the question as to why they do not adhere to each other within the normal nucleus. It has previously been shown (Glancy, 1940) that the chromosomes within the salivary gland nucleus are normally held apart by a thixotropic jelly matrix which becomes solated when the cell is torn. Although the chromosomes in the torn cell tend to become progressively sticky, this change is minimized if they are immediately removed from the cell so that contact with the torn cytoplasm is avoided. With prolonged contact, however, the chromosomes become so sticky that it is virtually impossible to manipulate them. The latter observations may indicate that the stickiness of isolated chromosomes is caused in part by chemical substances liberated from the disintegrating cytoplasm.

The reports of various investigators (Vonwiller and Audova, 1933; Barigozzi, 1938; Stefanelli, 1939; and Pfeiffer, 1940) to the effect that isolated chromosomes are tough, viscid gels, are in contrast to the observations in this paper which indicate them to be soft, easily deformable gels. The discrepancy may be explained in part by the failure of these investigators to observe precautions with respect to injured cytoplasm and the medium. My observations show that the consistency of isolated chromosomes tends to be greater than that of those within the nucleus, especially in the presence of injured cytoplasm. This increase in consistency seems to be associated with a simultaneous shrinkage of the chromosomes. Since the above investigators manipulated the chromosomes in the same drop of hemolymph or Ringer's solution in which the isolation had been performed, it is likely that the drop may have been acid because of the presence of the injured tissues. Even slight acidification of the medium has been shown to cause an increase in consistency. A similar effect results from the presence of calcium ions. Osmic acid vapors, used by Buck (1942) in treating the salivary glands prior to isolation of the chromosomes, cause far greater changes in physical properties although no shrinkage occurs. The findings of Buck, which we have confirmed using his technique, to the effect that the chromosomes are tough, cannot be compressed without buckling, and that they break squarely across when stretched, are at variance with those obtained on fresh chromosomes as shown in this investigation. It is thus evident that the consistency of the chromosomes is influenced by many factors.

The relative consistency of the bands and the interband regions has been discussed ever since Balbiani (1881) first suggested the bands to be gel and the interband regions, sol. Guareschi (1919) has observed that in dark field only the thicker bands are visible, whereas the thin bands and interband regions are not visible, from which he inferred that the former are in a gel state, and the latter in a weak gel, or more probably a sol state. Our microdissection experiments confirm Guareschi's inferences as to these differences in consistency. The thin bands and the interband regions must be weak gels rather than sols, however, for a puncture made in these regions leaves a temporary opening, a condition which does not obtain in a sol. This observation is further strengthened by the high elasticity of the chromosomes for, while gels are generally very elastic, a sol has negligible elasticity.

The extensibility and elasticity of the chromosomes have been studied by numerous investigators. Since it is well known that the properties of a colloidal structure

depend to a large extent on the medium, it is to be expected that the conditions under which the chromosomes are examined will influence their degree of elasticity. It is to be noted that the stretching experiments of Vonwiller and Audova (1933), Barigozzi (1938), Stefanelli (1939) and Pfeiffer (1940) were performed in 0.6 per cent NaCl, Ringer's solution, or hemolymph, without regard for the effects of the torn cytoplasm. These investigators found that the chromosomes could be stretched many times their length, though not reversibly. Pfeiffer and Stefanelli found stretching was not reversible beyond a 2 fold elongation as did Buck (1942) using osmicated chromosomes. It has been shown in this investigation that the elastic limit of fresh chromosomes in the intact cell is at least 5 fold, and when isolated in a KCl/NaCl medium, as much as 10 fold. This unusually high elasticity indicates the presence of long fibrous molecules (Mark, 1941) of which nucleoproteins are a characteristic type (Mirsky and Pollister, 1942).

The presence of a membrane on the salivary chromosomes has been described heretofore from the appearance of fixed and stained chromosomes (Schultz, 1941; Kodani, 1942). Metz (1934) believed the presence of a sheath or membrane to be necessary on theoretical grounds. Painter (1941) originally considered a pellicle to be present, but finally concluded, after studying the action of alkaline solutions on the salivary chromosomes, that the chromonemata are held together by a matrix, and that a pellicle is lacking. That the fresh chromosome possesses a membrane is strongly suggested by my microdissection experiments and observations that the outer boundary of the chromosome ruptures at a critical diameter when stretched by fluid injections. The membrane may aid in preventing contact between the chromosomes, since they tend to become sticky if it is torn, or it may serve to hold the chromonemata together.

The question as to whether or not the chromosomes in hyaline nuclei maintain their morphological integrity has frequently been raised. Stefanelli (1939) and Guareschi (1939), studying salivary gland nuclei rendered hyaline by immersion of the gland in hypotonic solutions, concluded that the chromosomes are completely dispersed. Stefanelli's conclusions, based primarily on the observations that microneedles can be moved back and forth within the hyaline nucleus without meeting resistance and that the nucleolus can be displaced without rebounding, do not conclusively demonstrate that the chromosomes are dispersed, however. The failure to find evidence for chromosomes by this technique might be explained in several ways: first, movement of the microneedles within the thixotropic nucleus might well induce solation; secondly, the consistency of the nuclear matrix in such nuclei may be close to that of the chromosomes; and thirdly, the swollen chromosomes are closely pressed against the nuclear membrane, leaving the center of the nucleus almost devoid of any resisting structure. To Guareschi's conclusions, it may be objected that a dilute gel cannot necessarily be distinguished from a sol under dark field illumination, and secondly, that the refractive indices of the colloidal particles in the chromosomes and the surrounding nuclear matrix may have been changed by the hydration which occurs in hypotonic solutions. Micro-injection results on salivary gland nuclei, likewise made hyaline by hypotonic solutions, indicate that the chromosomes, though invisible, maintain their morphological integrity, since injected carbon particles clearly indicated the outlines of the chromosome cylinders. If the chromosomes were completely dispersed, the injected carbon particles should be distributed at random in the nucleus.

The polytene concept of chromosome structure rather than the alveolar is supported by the various observations made in this investigation. The controversy regarding the structure of the salivary chromosomes results in part from the fact that fixed and stained chromosomes do not always present a similar appearance with regard to the finer details. Since the striations are especially evident in stretched chromosomes, Metz (1941) believes them to be artifacts produced by drawing out the walls of alveoli. Buck (1942) has provided experimental support for the alveolar hypothesis. It is agreed that a fibrillar-like appearance in homogeneous-appearing chromosomes can be produced by stretching. However, I have never observed a pre-existing alveolar structure in fresh chromosomes, nor does Buck state that the alveolar condition was present before he treated the glands with osmic acid vapors, although he does state that the treated chromosomes closely resemble those in the living animal. Most of his photomicrographs fail to show an alveolar structure. It is conceivable that stretching the chromosomes could cause aggregation of delicate, scarcely visible or non-visible fibrils, into the relatively coarse fibrils which are commonly seen in stretched chromosomes. Buck tried unsuccessfully to shred the chromosomes into fibrillae. This may easily be accounted for by the fact that he used osmicated chromosomes which are of such high consistency that shredding is impossible.

A fibrillar appearance was occasionally noted in the fresh salivary chromosomes studied in this investigation. Buck (1942) also reported that fresh chromosomes may occasionally show longitudinal striations, but he attached no particular significance to this observation. In chromosomes which show no structural details in the interband regions, a slight shrinkage produced by mild acidification (pH 6.5) results in the appearance of delicate longitudinal striations but never alveoli. Since the chromosomes have not been stretched, it does not seem likely that the fibrillar appearance can be explained on a basis of drawn out alveoli. The observation that fibrillae may appear at the broken ends of greatly stretched chromosomes may be interpreted in two ways, as Buck (1942) pointed out when he made a similar observation on osmicated chromosomes. The fibrillae may have been pre-existing, or they may be artifacts produced by the stretching. More significant is the microdissection from fresh unstretched chromosomes, either within the intact nucleus or after isolation, of delicate longitudinal fibrils which give the appearance of chromonemata. It is difficult to see how such fibrils could be removed from an alveolar mesh. Attempts to remove transverse fibrils, which should be equally possible if an alveolar structure exists, were uniformly unsuccessful. The finding that a fresh chromosome, expelled from a micropipette into Ringer's or KCl–NaCl solution, may break up into numerous delicate longitudinal fibrils, but never an alveolar mesh, is also difficult to explain except on a polytene basis.

## Summary and Conclusions

Micrurgical experiments performed on the fresh salivary gland chromosomes of mature Chironomus larvae within the nucleus of the intact cells and after their isolation into a specially designed medium indicate the following:

1. Puncture of the cell with a fine microneedle has little or no effect on the structure and physical properties of the chromosomes provided tearing of the cytoplasm is prevented.

2. Tearing of the cytoplasm causes the chromosomes to shrink markedly and become highly viscid.

3. The structure and physical properties of chromosomes isolated into a medium consisting of 0.09 M KCl, 0.06 M NaCl, and 0.005 M phosphate buffer at pH 7.0, are not appreciably altered, except for a slight increase in consistency, from those of chromosomes in the intact cell if precaution is taken to prevent contact of the chromosomes with the torn cytoplasm.

*Physical properties*

4. Evidence for stickiness of the chromosomes can be obtained only when the thixotropic jelly matrix surrounding them is solated.

5. The chromosomes are soft, easily deformable gels with interband regions of lower consistency than the bands.

6. The chromosomes are highly extensible and elastic. They can be stretched 5 fold within the intact cell without permanent deformation. Greater stretching cannot be accomplished without tearing the nuclear membrane. Isolated chromosomes regain their initial length after 10 fold elongation, but excessive stretching (12–25 ×) causes the chromosomes to break in an interband region.

7. Modifications in the properties of the chromosomes are easily induced by chemical agents. Dilute alkalies decrease consistency and elasticity, but increase extensibility. Dilute acids and calcium ions increase consistency, and decrease extensibility and elasticity. Osmic acid and formalin have a similar but much greater effect.

8. Chromosomes in nuclei hyalinized by hypotonic or alkaline Ringer's solution retain their morphological integrity.

*Structure*

9. The fresh chromosome possesses a delicate elastic membrane.

10. Chromosomes, either in the intact nucleus of the unoperated cell, or when isolated, occasionally display delicate longitudinal striations in the interband regions, but never show alveoli.

11. Slight acidification of the medium to pH 6.5 causes the appearance of similar striations in the interband regions of unstretched chromosomes.

12. Chromosomes broken in two by stretching frequently exhibit fibrillae at the broken ends.

13. Delicate fibrils may be dissected from the intact or isolated chromosome. The fibrils possess nodular swellings at intervals corresponding to the bands.

14. A chromosome drawn up into a micropipette of optimal size, then expelled into Ringer's or KCl–NaCl solution, may shred into numerous fibrillae.

These observations are best interpreted as supporting a polytene concept of chromosome structure.

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